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SUPPORTING INFORMATION

Radiosynthesis and characterisation of a GPR139 agonist

Sebastiaan Kuhne[a,b,#], Anne Cathrine Nøhr[a,#], Aleš Marek[c], Tomáš Elbert[c], Anders Bue Klein[a], Hans Bräuner-Osborne[a], Petrine Wellendorph[a], Daniel Sejer Pedersen[a,*]

[a] Department of Drug Design and Pharmacology, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark

[b] Amsterdam Institute for Molecules, Medicines and Systems (AIMMS), Division of Medicinal Chemistry, Faculty of Science, VU University Amsterdam, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands

[c] Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, 16610, Prague 6, Czech Republic

* Corresponding author. *E-mail address*: daniel.pedersen@sund.ku.dk

# These authors contributed equally.

Table of contents

REACTION PROGRESS OF REDUCTIVE DEBROMINATIONS OF 4 AND 5

The reaction was performed on a 30 mg scale.

Route 1. Reductive debromination of substrate **4**.

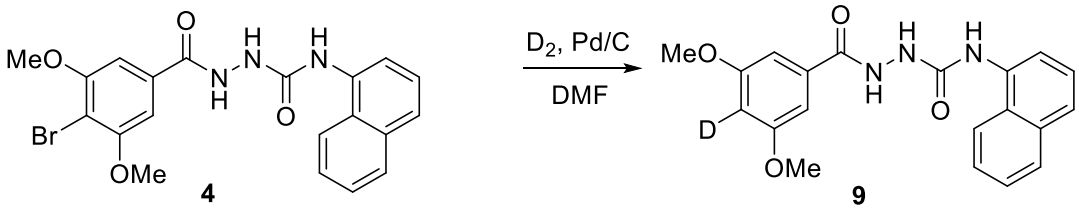
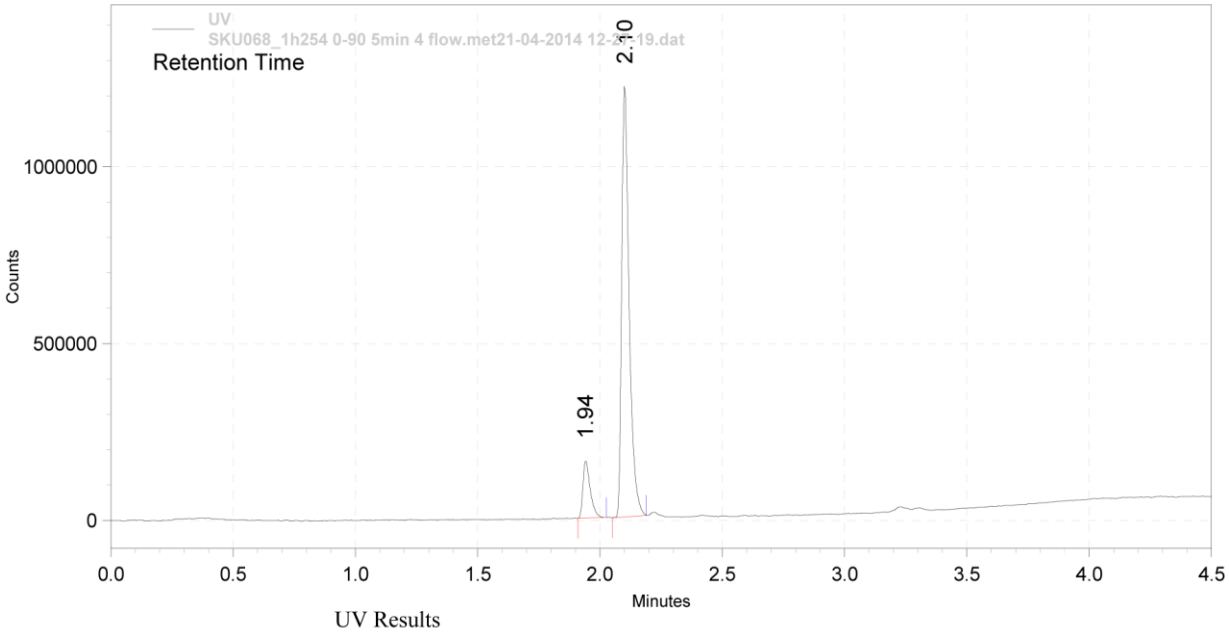


Table S1. Conversion of substrate **4** into **9**

Reaction time (h)	Conversion (%)[a]
1	12
2	17
3	23
5	34
21	88
30.5	> 95

[a] Conversion by integration of HPLC peaks detected at 254 nm.

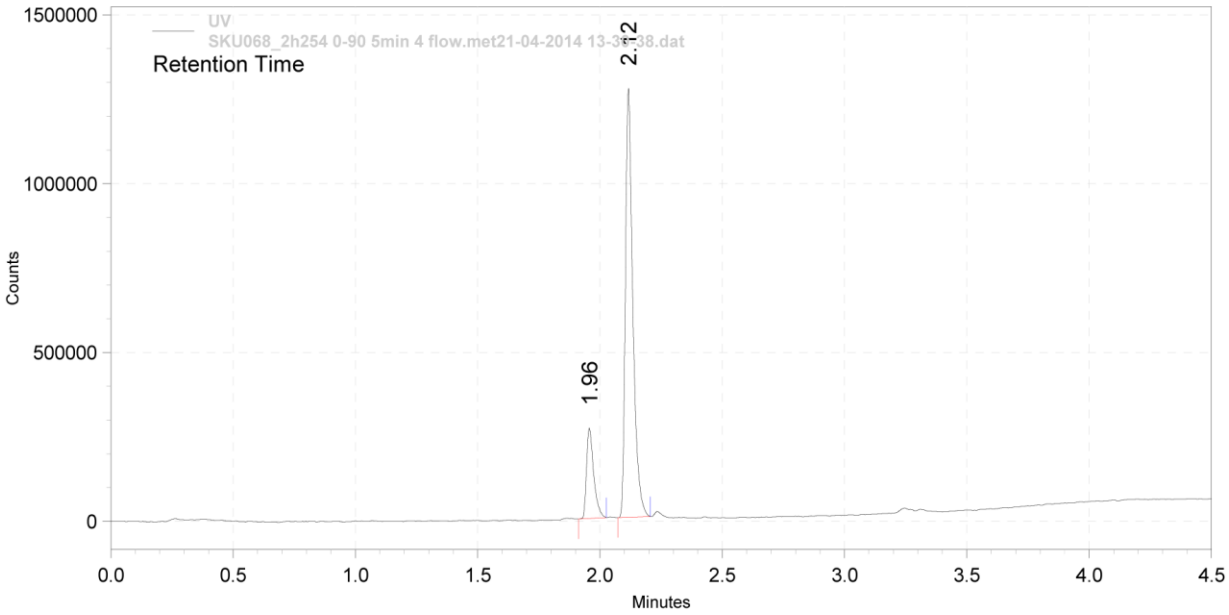
Reaction time: 1 h



UV Results

Peak no	RT	Area	Area Percent
1	1.94	334906	11.60
2	2.10	2552161	88.40

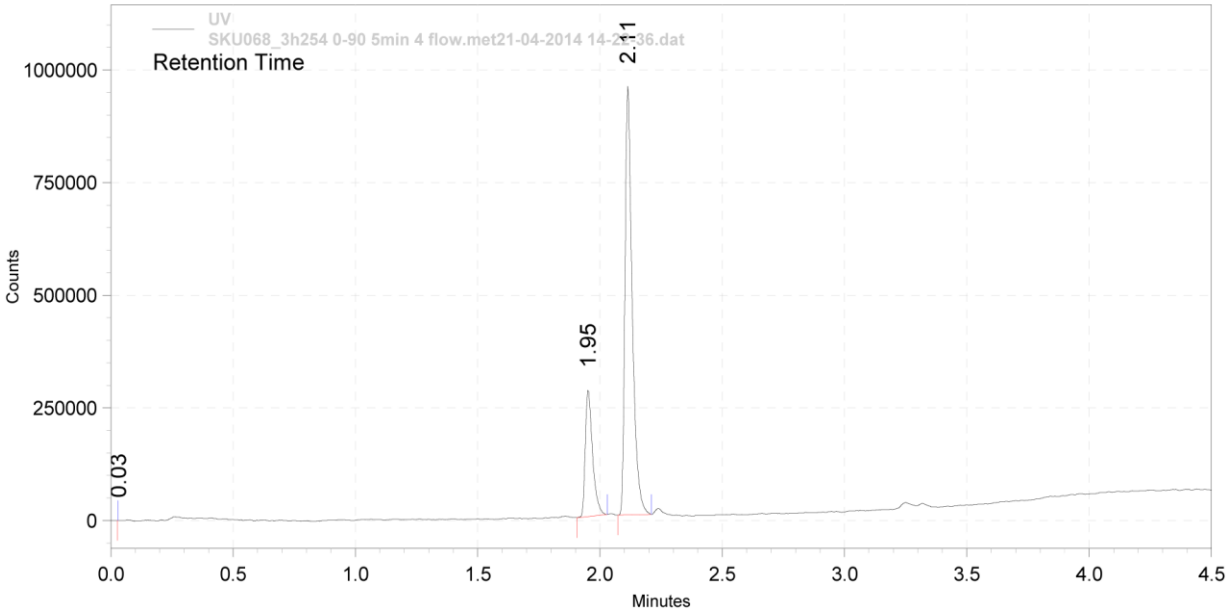
Reaction time: 2 h



UV Results

Peak no	RT	Area	Area Percent
1	1.96	538431	16.71
2	2.12	2683735	83.29

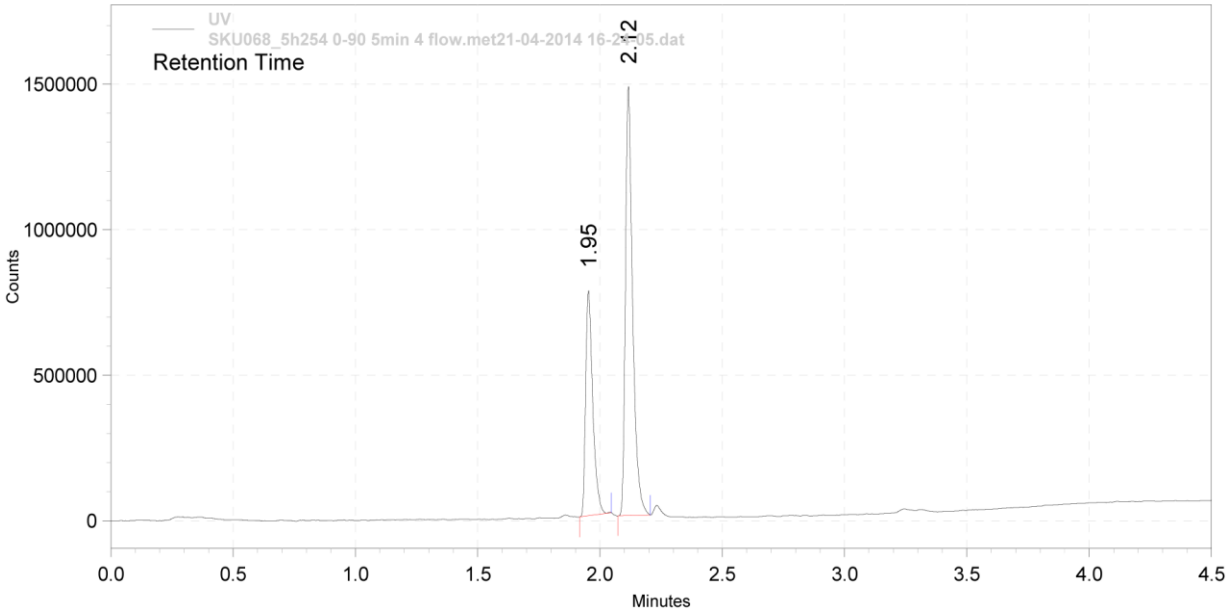
Reaction time: 3 h



UV Results

Peak no	RT	Area	Area Percent
1	0.03	0	0.00
2	1.95	572631	22.64
3	2.11	1957095	77.36

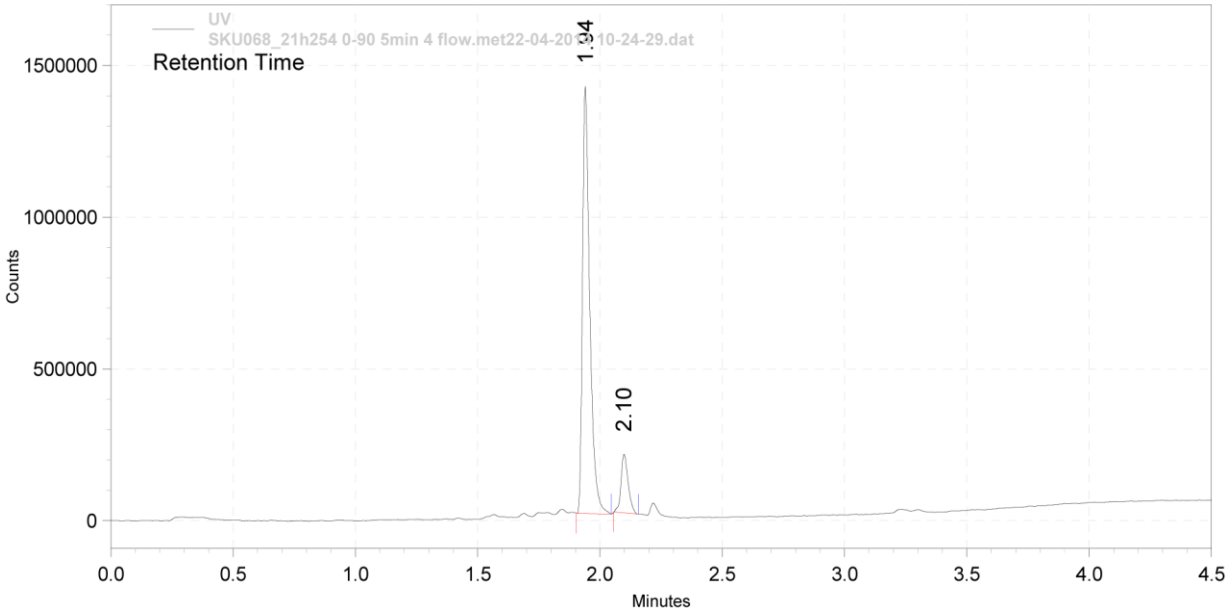
Reaction time: 5 h



UV Results

Peak no	RT	Area	Area Percent
1	1.95	1546462	33.83
2	2.12	3024730	66.17

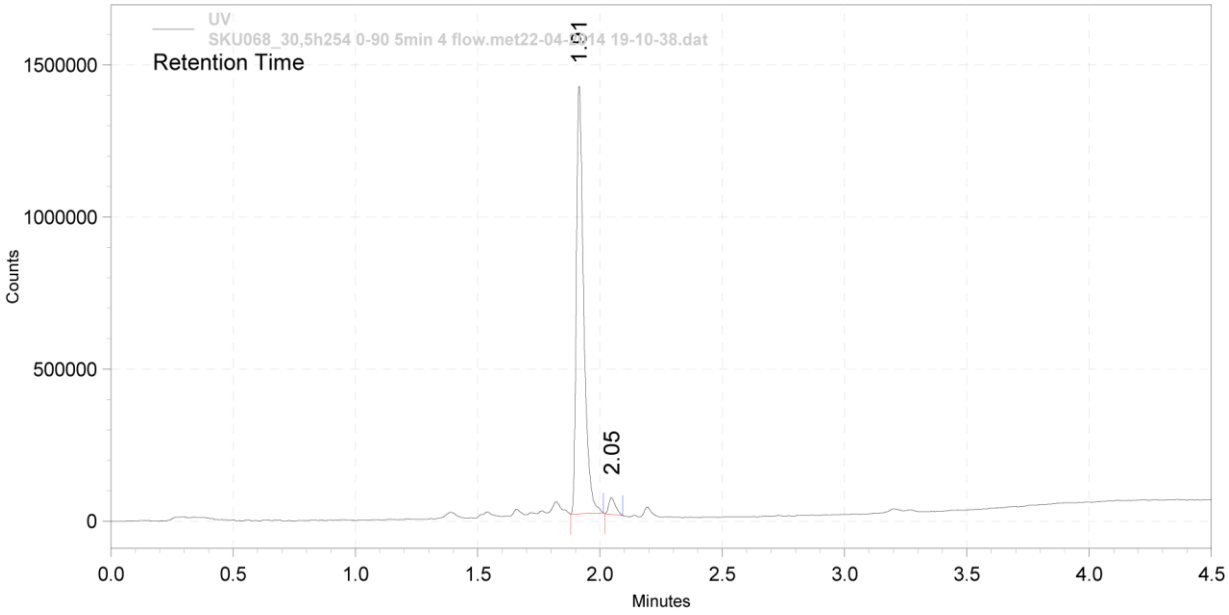
Reaction time: 21 h



UV Results

Peak no	RT	Area	Area Percent
1	1.94	2896786	88.19
2	2.10	387862	11.81

Reaction time: 30.5 h



UV Results

Peak no	RT	Area	Area Percent
1	1.91	2936376	96.55
2	2.05	105019	3.45

Route 2. Reductive debromination of substrate **5**.

The reaction was performed on a 30 mg scale.

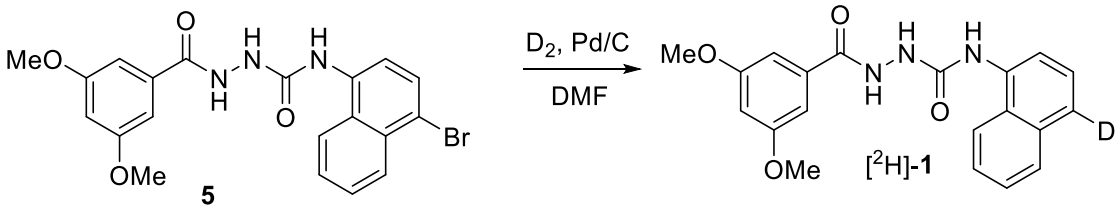
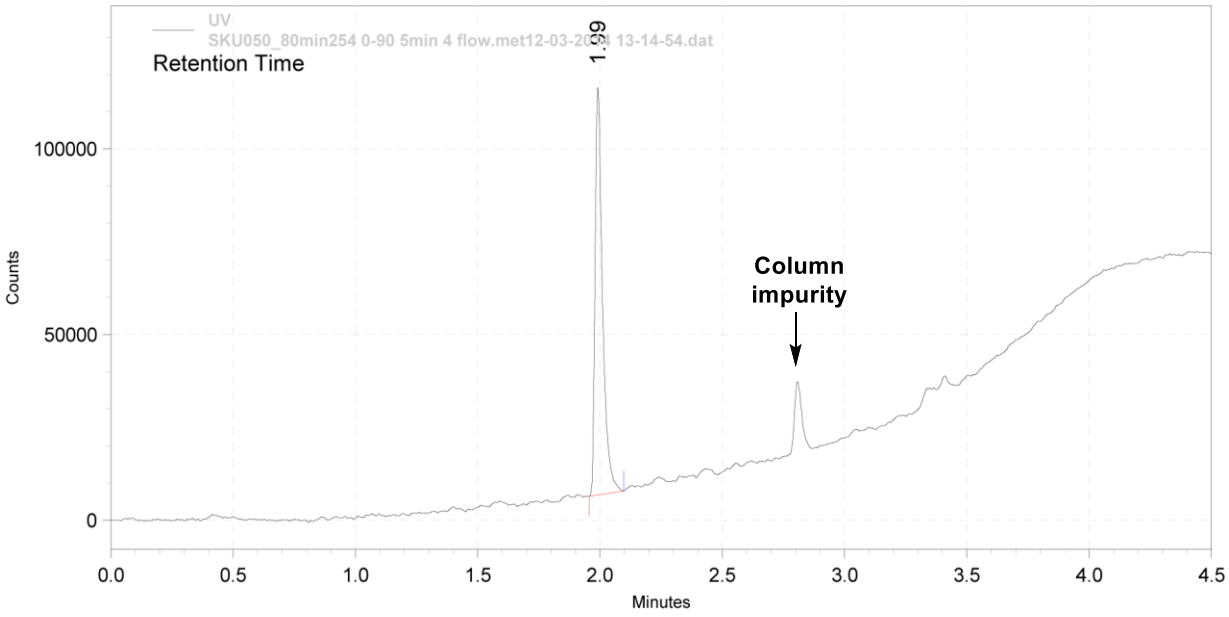


Table S2. Conversion of substrate **5** into [^{2}H]-**1**

Reaction time (h)	**Conversion (%)**[a]
1.33	> 95

[a] Conversion by integration of HPLC peaks detected at 254 nm.

Reaction time: 1.33 h



UV Results Peak no	RT	Area	Area Percent
1	1.99	233577	100.00

CHEMISTRY EXPERIMENTALS

General information

All reactions were carried out under nitrogen or argon. Solvents were of chromatography grade and THF and DMF were dried using an SG Water solvent purification system. Commercially acquired chemicals were used without further purification. Chemical names were generated using ChemBioDraw Ultra 14.0.0.117.

Thin-Layer Chromatography (TLC)

TLC analysis were performed using Merck pre-coated silica gel 60 F_{254} plates and visualised using UV. Retention factor, R_f, values were rounded to the nearest 0.05.

Fourier Transform Infrared Spectroscopy (FT-IR)

FT-IR was recorded neat on a Perkin-Elmer Spectrum One IR spectrometer with a universal ATR accessory and the signals are reported in wavenumbers (cm^{-1}).

Nuclear Magnetic Resonance Spectroscopy (NMR)

NMR spectra were recorded on a Bruker 300 (300 MHz), Bruker 400 (400 MHz) or Bruker 600 (600 MHz) instrument. Signals are reported in ppm (δ), followed by integral, multiplicity (s = singlet, d = doublet, t = triplet, br s = broad signal and m = multiplet) and coupling constant, J (in Hz and rounded to the nearest 0.5 Hz). NMR solvents were used as internal standard (δ ^{1}H NMR: $CDCl_3$ 7.26; DMSO-d_6 2.50. δ ^{13}C NMR: $CDCl_3$ 77.16; DMSO-d_6 39.52). Coupling constants (J) are given in Hertz (Hz) and rounded to the nearest 0.5 Hz.

High Performance Liquid Chromatography (Mass Spectrometry) (HPLC-MS)

Method A: LC analysis was carried out on a Merck Hitachi LaChrom HPLC system using a Chromolith® SpeedROD RP-18 column (2 µm, 50 × 4.6 mm) with UV detection at 254 nm. Mobile phase (MP) A: 0.1% trifluoroacetic acid (TFA), 100% H_2O (v/v). MP B: 0.1% TFA, 10%

H_2O in 90% MeCN (v/v). Flow rate: 4 mL/min. Gradient: 0-3.5 min: 0-100% MP B; 3.5-4.2 min: 100% MP B; 4.2-5.2 min 100% MP A.

Method B: Low resolution mass spectrometry was recorded on a Bruker Esquire 3000 plus instrument connected to an Agilent 1200 HPLC system, using an Electrospray Ionization (ESI) mass detector. HPLC-MS was carried out on an Agilent 1200 series system using an Xbridge RP C18 column (3.5 µm, 100 × 4.6 mm) with UV detection at 254 nm. Mobile phase (MP) A: 0.2% HCOOH, 99.8% H_2O (v/v). Mobile phase B: 0.2% HCOOH, 99.8% MeCN (v/v). Flow rate: 0.5 ml/min. Gradient: 0-7 min: 0-90% MP B, 7-9 min: 90% MP B, 9-16 min: 10% MP B.

Method C: Identification was done by UV detection at 215 and 245 nm. Column: Synergi 4µ POLAR RP80 (250mm × 4.6 mm, 10 mm). Flow: 1 mL/min. Eluents: (A) 99.9% purified water, 0.1% TFA; (B) 99.9% acetonitrile, 0.1% TFA. Gradient: 0–15 min, 80% A, 20% B; 15–25 min, 80-40% A, 20-60% B; 25–30 min, 35 A%, 65% B; 30-35 min, 35-0% A; 65-100% B. The retention time of [^{2}H]-**1** product was detected at 18.3 min (matched with [^{1}H]-**1**), substrate **5** expected at 20.4 min was not detected.

Method D: Identification was done by UV detection at 215 and 245 nm. Column: Synergi 4µ POLAR RP80 (250mm × 4.6 mm, 10 mm). Flow: 4.7 mL/min. Eluents: (A) 99.9% purified water, 0.1% TFA; (B) 99.9% acetonitrile, 0.1% TFA. Gradient: 0–5 min, 80% A, 20% B; 5–20 min, 80-40% A, 20-60% B; 20–30 min 40-35 A%, 60-65% B; 30-35 min, 35% A; 65% B, 35-40 min, 35-0% A; 65-100% B. The retention time of [^{3}H]-**1** product was detected at 22.0 min.

High Resolution Mass Spectrometry (HRMS)

High resolution mass spectra were recorded on a Micromass Q-TOF 1.5, UB137 or on a time of flight (TOF) MS system, coupled to an analytical HPLC and ESI detector. HRMS HPLC was performed on a C18 column (25 cm × 4.6 mm, 5 μm) with a linear gradient (10 % to 100 % MeOH in H_2O, containing 0.1 % TFA, in 20 min, v/v) at a flow rate of 1 ml/min and UV detection at 215 nm.

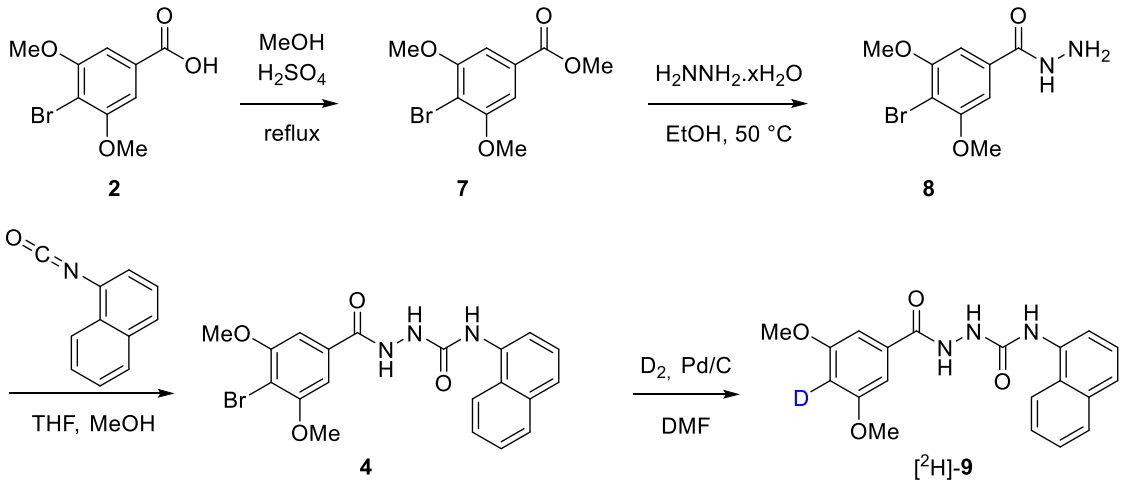
Equipment for tritium chemistry

The tritium experiment was carried out in a glove box equipped with a Tritiation manifold from RC-TRITEC AG (Teufen, Switzerland) suitable for handling 100 to 1000 Ci of carrier-free tritium gas. Model deuterium reactions were carried out on a deuteration manifold of similar construction as the tritiation manifold. The ^{3}H-labeled product was analysed and purified on a semi-preparative radio-HPLC [pump Waters 600, UV detector Waters 2487, radio-detector Ramona with analytical cell (LSC) and solid scintilator preparative cell (Raytest, Germany), data management software Empower 2 from Waters]. ^{3}H NMR spectra were recorded on a spectrometer Bruker Avance II 300 MHz. Solvents and volatile impurities were evaporated on a LABCONCO CentriVap. ^{3}H-Activities were determined on a liquid scintillation spectrometer PerkinElmer TriCarb 2900TR. The mass spectra were obtained in the ESI mode either on a Q-Tof micro from Waters or on an LTQ Orbitrap XL from Thermo Fisher Scientific for the HRMS spectra.

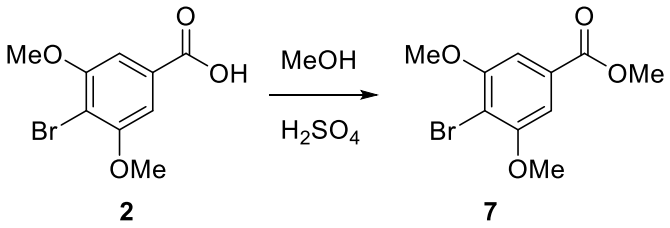
Calculation of specific activity for [^{3}H]-1

The tritium enrichment at the 4-position of the naphtalene moiety of radioligand [3H]-1 was determined to 67% by ^{1}H NMR, corresponding to 19.3 Ci/mmol).

Synthetic route 1



Methyl 4-bromo-3,5-dimethoxybenzoate (7)



2 **7**

4-Bromo-3,5-dimethoxybenzoic acid (5.00 g, 19 mmol) was suspended in MeOH (40 ml) and H_2SO_4 (0.125 ml) was added. The resulting mixture was stirred for 16 hours at reflux. The mixture was cooled to ambient temperature. A white solid was formed upon cooling and the excess MeOH was evaporated *in vacuo*. The solid was taken up in CH_2Cl_2 (75 ml) and saturated $NaHCO_3$ solution (100 ml) was added. The aqueous phase was extracted with CH_2Cl_2 (3 × 75 ml). The organic layers were combined and washed with water, dried over Na_2SO_4, filtered and evaporated *in vacuo* to afford methyl ester **7** (4.46 g, 84%) as a white solid.

TLC R_f = 0.35 (*n*-heptane: CH_2Cl_2 8:2, v/v);

HPLC R_T = 2.13 min (method A);

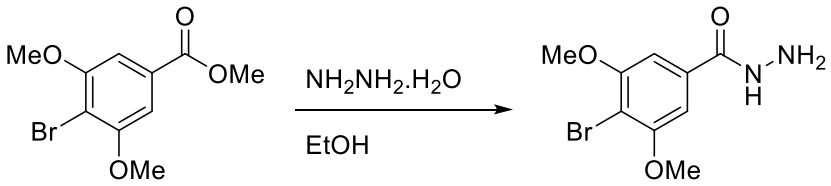
IR (neat) ν_{max} = 2956, 1709, 1228, 1115 cm^{-1};

^{1}H NMR (600 MHz, DMSO-*d6*): δ 7.23 (s, 2H), 3.91 (s, 6H), 3.88 (s, 3H);

^{13}C NMR (151 MHz, DMSO-*d6*): δ 165.6, 156.6, 130.0, 105.6, 105.2, 56.6, 52.5;

HRMS *m/z* (ESI+) found: 296.9740 $[M+Na]^+$; $C_{10}H_{11}BrO_4Na^+$ requires *M*, 296.9733.

4-Bromo-3,5-dimethoxybenzohydrazide (8)



7 **8**

Methyl ester **7** (2.00 g, 7.27 mmol) was dissolved in EtOH (30 ml). Subsequently, hydrazine hydrate (5.3 ml) was added and the resulting mixture was stirred for 8 hours at 50 °C. The reaction was cooled to room temperature and a white precipitate was formed. The white precipitate was filtered off, washed with EtOH and dried *in vacuo* to afford hydrazide **8** (1.15 g, 57%) as a white solid which was used in the next step without further purification.

TLC R_f = 0.2 (3% MeOH in CH_2Cl_2, v/v);

HPLC R_T = 1.37 min (method A);

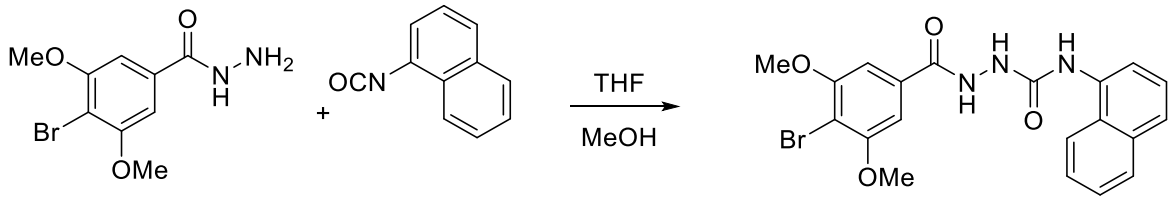
IR (neat) ν_{max} = 3311, 1579, 1237, 1116 cm^{-1};

^{1}H NMR (600 MHz, DMSO-d_6): δ 9.87 (br s, 1H), 7.18 (s, 2H), 4.52 (d, J = 4 Hz, 2H), 3.88 (s, 6H);

^{13}C NMR (151 MHz, DMSO-d_6): δ 164.8, 156.3, 133.7, 103.5, 102.7, 56.5;

HRMS *m/z* (ESI+) found: 296.9854 $[M+Na]^+$; $C_9H_{11}BrN_2O_3Na^+$ requires M, 296.9845.

2-(4-Bromo-3,5-dimethoxybenzoyl)-*N*-(naphthalen-1-yl)hydrazine-1-carboxamide (4)



8 **4**

By a method adapted from Shi *et al.*[1] hydrazide **8** (325 mg, 1.18 mmol) was dissolved in anhydrous THF (7.5 ml) and MeOH (3 ml). Subsequently, 1-isocyanatonaphthalene (200 mg, 1.18 mmol) suspended in anhydrous THF (2 ml) was added and a white precipitate was formed. After stirring for 1 hour at ambient temperature the precipitate was filtered off and washed with CH_2Cl_2 (approx. 5 ml). The solid was dried *in vacuo*. Finally, the solid was suspended in CH_2Cl_2 and evaporated to dryness and dried *in vacuo* to give carboxamide **4** (410 mg, 78%) as a white amorphous solid.

TLC R_f = 0.3 (3% MeOH in CH_2Cl_2, v/v);

HPLC R_T = 2.07 min (method A);

IR (neat) ν_{max} = 3344, 1581, 1529, 1121 cm^{-1};

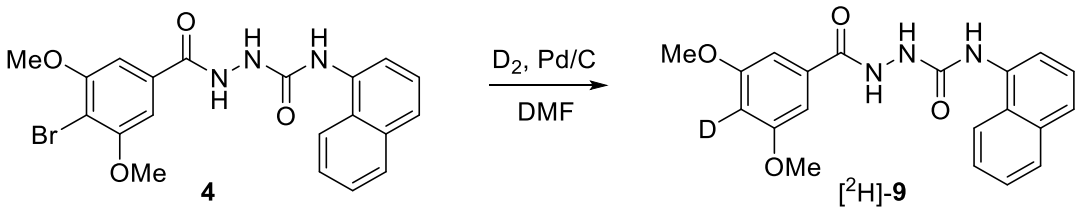
^{1}H NMR (600 MHz, DMSO-d_6): δ 10.54 (s, 1H), 8.96 (s, 1H), 8.53 (s, 1H), 8.10 (d, J = 8.5 Hz, 1H), 7.95 – 7.91 (m, 1H), 7.82 (br s, 1H)*, 7.68 (d, J = 8 Hz, 1H), 7.60 – 7.52 (m, 2H), 7.47 (t, J = 8 Hz, 1H), 7.30 (s, 2H), 3.92 (s, 6H);

^{13}C NMR (151 MHz, DMSO-d_6): δ 165.5, 156.4, 156.2 (br s), 134.3, 133.7, 132.9, 128.2, 127.0 (br s), 125.9, 125.8, 125.6, 123.7 (br s), 122.0 (br s), 118.7 (br s), 104.1, 103.5, 56.6;

HRMS *m/z* (ESI+) found: 466.0375 $[M+Na]^+$; $C_{20}H_{18}N_3O_4BrNa^+$ requires *M*, 466.0373.

* At 7.82 ppm in ^{1}H NMR a broad peak was observed. High temperature NMR experiments showed a doublet.

2-(3,5-Dimethoxybenzoyl-4-[$^2H_1$])-*N*-(naphthalen-1-yl)hydrazine-1-carboxamide ([^{2}H]-9)



Carboxamide **4** (30 mg, 0.068 mmol) was dissolved in anhydrous DMF (2.5 ml) and the round bottom flask (100 ml) was evacuated and backfilled with N_2 several times. Subsequently, Pd/C (loading 10 wt. %, 9.8 mg) was added and the flask was evacuated and backfilled first with N_2 followed by D_2. The reaction mixture was stirred at ambient temperature for 30.5 hours. The reaction mixture was diluted with DMF and filtered through a plug of Celite (the Celite was pre-washed with DMF). The Celite was washed with DMF (50 ml) and the organic layer was concentrated *in vacuo* (water bath 55 °C) to yield the crude product as an orange oil (contaminated with DMF). MeOH and Et_2O were added to the crude product and a pink precipitate was formed, filtered off and washed with additional Et_2O. The filtrate was evaporated *in vacuo*. Et_2O and MeOH were added and a white solid was formed, which was filtered off and dried under high vacuum to give the crude reduced product (9 mg, 36%) as a white solid. ^{1}H NMR showed that the product was a mixture of D and H-reduced products [^{2}H]-**9** and **1** ([^{2}H]-**9**:**1** ratio approx. 4:1 based on the ^{1}H NMR resonance observed at 6.70 ppm).

HPLC R_T = 7.84 min (method B);

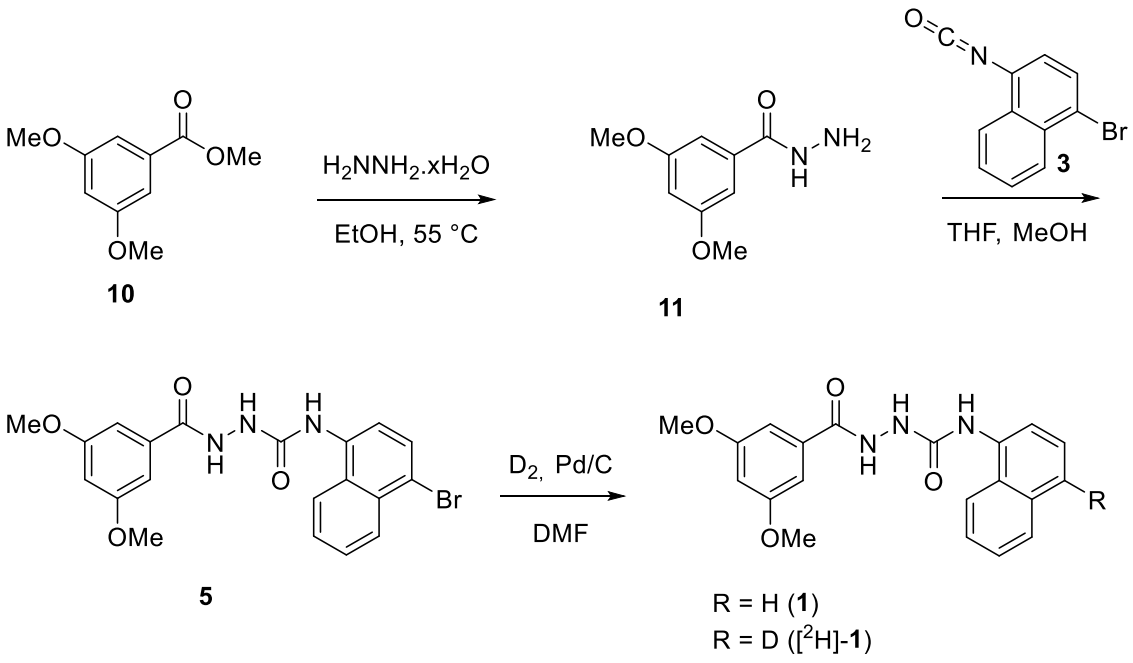
1: HRMS *m/z* (ESI+) found: 388.1285 $[M+Na]^+$; $C_{20}H_{19}N_3O_4Na^+$ requires *M*, 388.1268.

9: HRMS *m/z* (ESI+) found: 389.1325 $[M+Na]^+$; $C_{20}H_{18}DN_3O_4Na^+$ requires *M*, 389.1331.

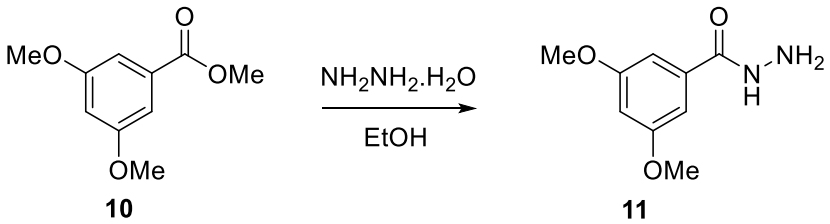
It must be noted that the reductive debromination reaction with substrate **5** on a 30 mg scale to obtain [^{2}H]-**1** was performed under identical conditions. ^{1}H NMR showed that the product was a

mixture of D and H-reduced products [^{2}H]-**1** and **1** ([^{2}H]-**1**:**1** ratio approx. 1:1 based on the ^{1}H NMR resonance observed at 7.67 ppm.

Synthetic route 2



3,5-Dimethoxybenzohydrazide (11)



10 **11**

Methyl 3,5-dimethoxybenzoate **10** (4.00 g, 20.4 mmol) was dissolved in EtOH (100 ml). Subsequently, hydrazine hydrate (15 ml) was added and the resulting mixture was stirred for 8 hours at 55 °C. The reaction was cooled to room temperature and a white precipitate was formed. The precipitate was filtered off, washed with EtOH (150 ml) and dried *in vacuo*. The solid was recrystallised from EtOH to afford hydrazide **11** (1.31 g, 32%) as a white crystalline solid.

TLC R_f = 0.35 (3% MeOH in CH_2Cl_2, v/v);

HPLC R_T = 1.19 min (method A);

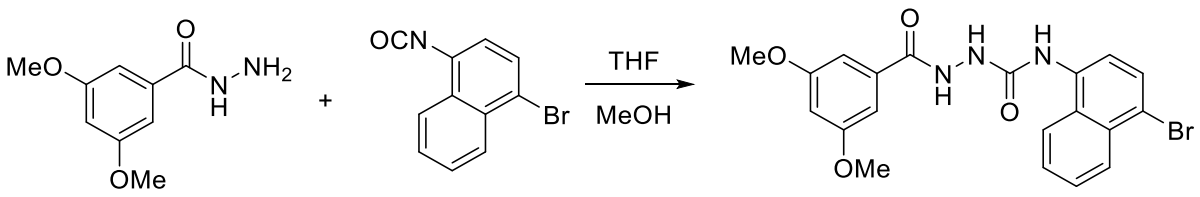
IR (neat) ν_{max} = 3344, 2945, 1589, 1526, 1154 cm^{-1};

^{1}H NMR (600 MHz, DMSO-d_6): δ 9.72 (s, 1H), 6.98 (d, J = 2.5 Hz, 2H), 6.62 (t, J = 2.5 Hz, 1H), 4.47 (s, 2H), 3.77 (s, 6H);

^{13}C NMR (151 MHz, DMSO-d_6): δ 165.3, 160.3, 135.3, 104.8, 103.0, 55.4;

HRMS *m/z* (ESI+) found: 219.0729 $[M+Na]^+$; $C_9H_{12}N_2O_3Na^+$ requires M, 219.0740.

***N*-(4-Bromonaphthalen-1-yl)-2-(3,5-dimethoxybenzoyl)hydrazine-1-carboxamide (5)**



11 **3** **5**

By a method adapted from Shi *et al.*[1] hydrazide **11** (166 mg, 0.85 mmol) was dissolved in anhydrous THF (8 ml) and MeOH (2 ml). Subsequently, 1-bromo-4-isocyanatonaphthalene (200 mg, 0.81 mmol) suspended in anhydrous THF (2.5 ml) was added and a white precipitate immediately started to form. After stirring for 80 minutes at ambient temperature the precipitate was filtered off and washed with CH_2Cl_2 (approx. 5 ml). The solid was suspended in CH_2Cl_2:THF and evaporated to dryness, followed by suspension in THF and evaporation to dryness. Finally, the solid was suspended in CH_2Cl_2, evaporated to dryness (twice) and dried *in vacuo* to give carboxamide **5** (290 mg, 81%) as a white amorphous solid.

TLC R_f = 0.45 (3% MeOH in CH_2Cl_2, v/v);

HPLC R_T = 2.23 min (method A);

IR (neat) ν_{max} = 3388, 3321, 3180, 1595, 1550, 1206 cm^{-1};

^{1}H NMR (600 MHz, DMSO-*d*6) δ 10.39 (s, 1H), 9.04 (s, 1H), 8.52 (s, 1H), 8.20 – 8.12 (m, 2H), 7.85 (d, J = 8.1 Hz, 1H), 7.82 – 7.66 (m, 3H)*, 7.11 (d, J = 2.3 Hz, 2H), 6.70 (t, J = 2.3 Hz, 1H), 3.81 (s, 6H);

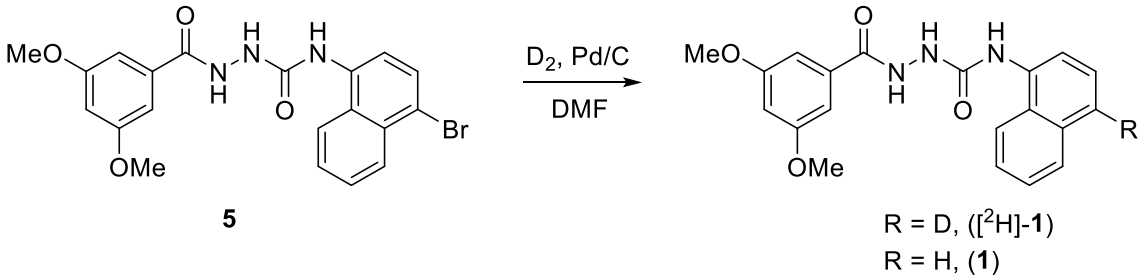
^{13}C NMR (151 MHz, DMSO-d_6): δ 165.9, 160.3, 156.1 (br s), 134.8, 134.5, 131.4, 129.8, 128.0 (br s), 127.8, 126.8, 126.6, 122.9 (br s), 119.1 (br s), 116.2 (br s), 105.4, 103.7, 55.5;

HRMS *m/z* (ESI+) found: 466.0388 $[M+Na]^+$; $C_{20}H_{18}N_3O_4BrNa^+$ requires *M*, 466.0373.

* At 7.81 ppm in ^{1}H NMR a broad peak was observed. High temperature NMR experiments showed a doublet.

All analytical data was consistent with that previously reported.[1]

2-(3,5-Dimethoxybenzoyl)-*N*-(naphthalen-1-yl-4-[$^2H_1$])hydrazine-1-carboxamide ([^{2}H]-1)



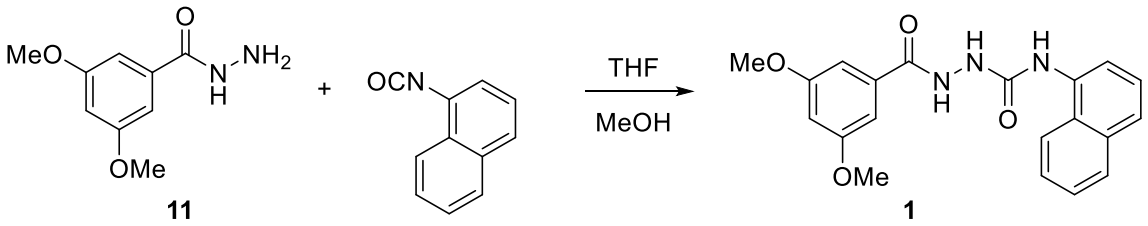
Carboxamide **5** (100 mg, 0.225 mmol) was dissolved in anhydrous DMF (7.5 ml) and the round bottom flask (100 ml) was evacuated and backfilled with N_2 several times. Subsequently, Pd/C (loading 10 wt. %, 30 mg) was added and the flask was evacuated and backfilled first with N_2 followed by D_2. The reaction mixture was stirred at ambient temperature for 8 hours, however no further conversion of starting material was observed by HPLC after 5 hours despite the addition of additional Pd/C (6 mg). It should be noted that the conversion after 1 hour was ~ 93% and after 2 hours it was > 95%, which is essentially identical to that observed in the initial small scale screening reaction (30 mg). The reaction mixture was diluted with DMF (10 ml) and filtered through a plug of Celite (the Celite was pre-washed with DMF). The Celite was washed with DMF (40 ml) and the organic layer was concentrated *in vacuo* (water bath 55 °C) to yield the crude product as an orange oil (0.25 g, contaminated with DMF). MeOH was added to the crude product and a precipitate was formed. The precipitate was filtered off, washed with MeOH and dried under high vacuum to give the pure reduced product (20 mg, 24%) as a pale orange solid. As anticipated ^{1}H NMR showed that the product was a mixture of D and H-reduced products [^{2}H]-**1** and **1** ([^{2}H]-**1**:**1** ratio approx. 2:3 based on the ^{1}H NMR resonance observed at 7.67 ppm).

HPLC R_T = 7.81 min (method B);

1: HRMS *m/z* (ESI+) found: 388.1252 $[M+Na]^+$; $C_{20}H_{19}N_3O_4Na^+$ requires *M*, 388.1268.

$[^2H]$-**1: HRMS** *m/z* (ESI+) found: 389.1333 $[M+Na]^+$; $C_{20}H_{18}DN_3O_4Na^+$ requires *M*, 389.1331.

2-(3,5-Dimethoxybenzoyl)-*N*-(naphthalen-1-yl)hydrazine-1-carboxamide (1)



By a method adapted from Shi *et al.*[1] hydrazide **11** (104 mg, 0.53 mmol) was dissolved in anhydrous THF (4 ml) and MeOH (2.5 ml). Subsequently, 1-isocyanatonaphthalene (85 mg, 0.50 mmol) dissolved in anhydrous THF (1 ml) was added and a white precipitate was formed. The resulting mixture was stirred for 1.5 hours at ambient temperature. The white precipitate was filtered off, washed with THF (approx. 2 ml) and dried *in vacuo*. The solid was suspended in CH_2Cl_2 and evaporated to dryness (three times) and dried *in vacuo* to give carboxamide **1** (128 mg, 70%) as a white amorphous solid.

TLC R_f = 0.4 (3% MeOH in CH_2Cl_2, v/v);

HPLC R_T = 7.82 min (method B);

IR (neat) ν_{max} = 3276, 1597, 1207, 1158 cm^{-1};

^{1}H NMR (600 MHz, DMSO-d_6): δ 10.37 (s, 1H), 8.91 (s, 1H), 8.46 (s, 1H), 8.10 (d, J = 8.5 Hz, 1H), 7.95 – 7.91 (m, 1H), 7.81 (br s, 1H)*, 7.67 (d, J = 8 Hz, 1H), 7.60 – 7.50 (m, 2H), 7.47 (t, J = 8 Hz, 1H), 7.11 (d, J = 2 Hz, 2H), 6.70 (t, J = 2 Hz, 1H), 3.81 (s, 6H);

^{13}C NMR (151 MHz, DMSO-d_6): δ 165.9, 160.3, 156.3 (br s), 134.5, 134.3, 133.7, 128.2, 126.9 (br s), 125.9, 125.7, 125.6, 123.7 (br s), 122.0 (br s), 118.8 (br s), 105.4, 103.7, 55.5;

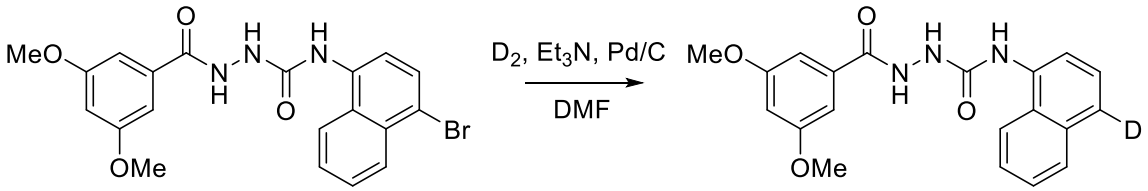
HRMS *m/z* (ESI+) found: 388.1267 $[M+Na]^+$; $C_{20}H_{19}N_3O_4Na^+$ requires *M*, 388.1268.

* At 7.81 ppm in ^{1}H NMR a broad peak was observed. High temperature NMR experiments showed a doublet.

All analytical data was consistent with that previously reported.[1]

Optimised deuteration conditions

2-(3,5-Dimethoxybenzoyl)-*N*-(naphthalen-1-yl-4-[$^2H_1$])hydrazine-1-carboxamide ([^{2}H]-1)



5 [^{2}H]-**1**

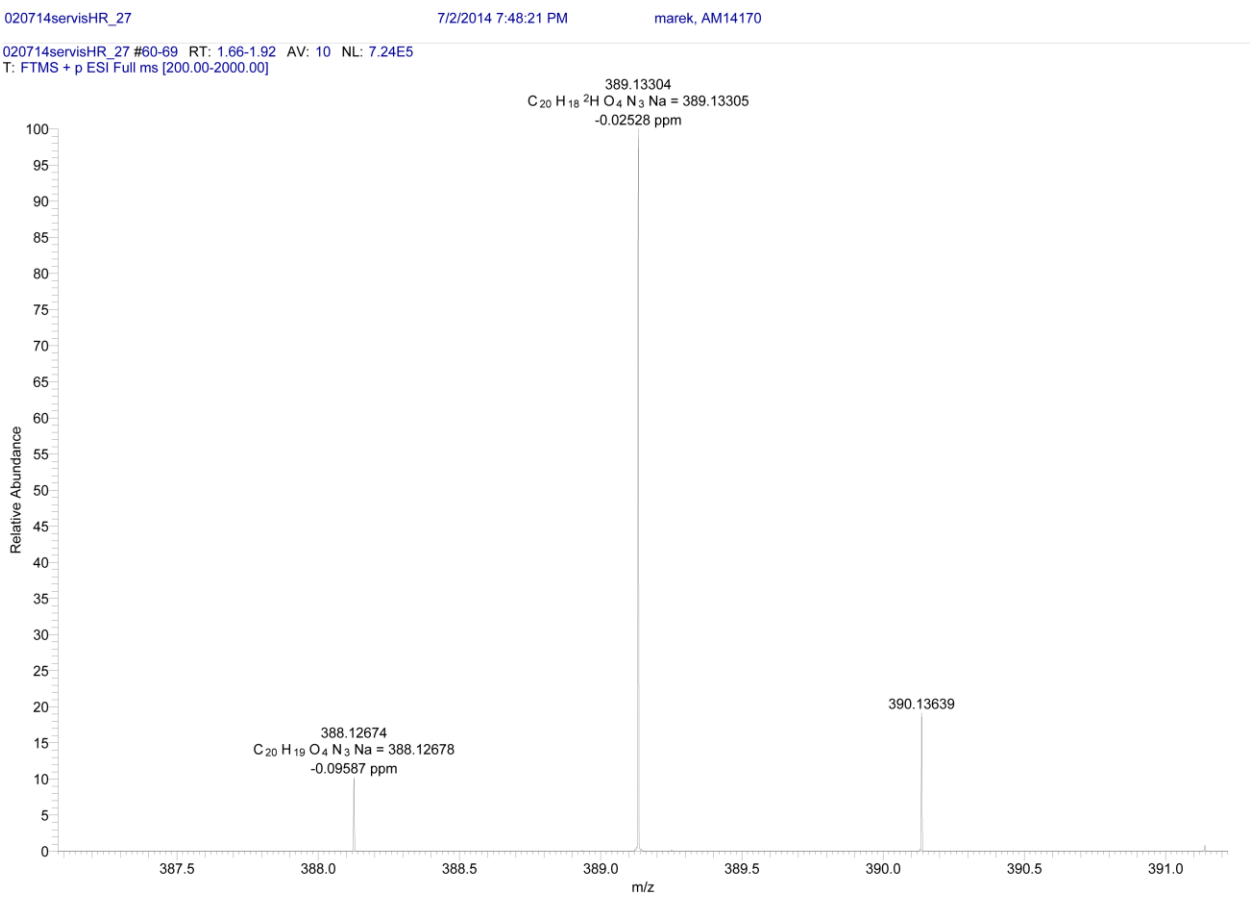
Carboxamide **5** (5 mg, 11 µmol), Pd/C (loading 30 wt. %, 5 mg), triethylamine (5 µl, 33 µmol) and DMF (1 ml) were placed in a round-bottomed deuteration flask (2 ml). The flask was mounted on the deuterium manifold and the reaction mixture was degassed by three successive freeze-thaw cycles (liquid nitrogen, vacuum of oil pump). The reaction mixture was stirred under D_2 (900 mbar) for 1 hour at room temperature. The mixture was cooled by liquid nitrogen and the D_2 was disposed of under vacuum. The reaction mixture was filtered through a 0.45 µ PTFE syringe filter and the deuteration flask and filter were washed by three 1 ml portions of DMF. A sample of the reaction mixture was analysed by HPLC showing full conversion of starting material and solely the desired product. The DMF was evaporated on the Centrivap to give crude [^{2}H]-**1** as a white solid (5 mg, >80%). The crude product was used for ^{1}H NMR and MS analysis with no further purification. As anticipated ^{1}H NMR showed that the product was a mixture of D- and H-reduced products [^{2}H]-**1** and **1** ([^{2}H]-**1**:**1** ratio approx. 4:1 based on the ^{1}H NMR resonance observed at 7.67 ppm). ESI-HRMS data confirmed high enrichment of deuterium ([^{2}H]-**1**:**1** ratio approx. 85:15 based on the intensities of appropriate signals, see below).

HPLC R_T = 18.3 min (method C), substrate **5** expected at 20.4 min was not detected.

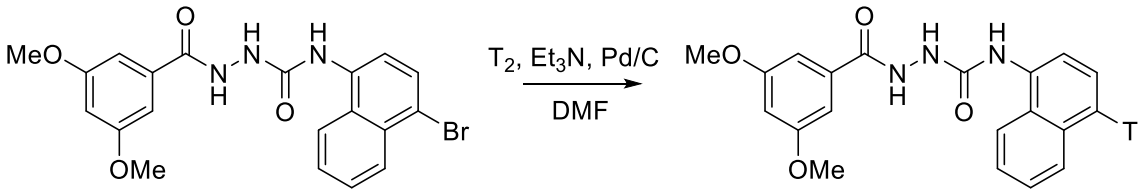
MS (m/z): 367.1 [M+1], 389.1 [M+Na], 755.3 [2M+Na].

1: HRMS *m/z* (ESI+) found: 388.1267 $[M+Na]^+$; $C_{20}H_{19}N_3O_4Na^+$ requires *M*, 388.1268.

[^{2}H]-**1: HRMS** *m/z* (ESI+) found: 389.1330 $[M+Na]^+$; $C_{20}H_{18}DN_3O_4Na^+$ requires *M*, 389.1331.


020714servisHR_27 7/2/2014 7:48:21 PM marek, AM14170
020714servisHR_27 #60-69 RT: 1.66-1.92 AV: 10 NL: 7.24E5
T: FTMS + p ESI Full ms [200.00-2000.00]
389.13304
C20 H18 2H O4 N3 Na = 389.13305
-0.02528 ppm
388.12674
C20 H19 O4 N3 Na = 388.12678
-0.09587 ppm
390.13639
Relative Abundance
m/z

2-(3,5-Dimethoxybenzoyl)-*N*-(naphthalen-1-yl-4-[$^3H_1$])hydrazine-1-carboxamide



5 [^{3}H]-**1**

The synthetic precursor **5** (5 mg, 11 µmol), Pd/C (loading 30 wt. %, 5 mg), triethylamine (5 µl, 33 µmol) and DMF (1 ml) were placed in a round-bottomed flask (2 ml). The flask was mounted on a tritium manifold placed in a glove box and the reaction mixture was degassed by three successive freeze-thaw cycles (liquid nitrogen, vacuum of turbopump). Career free T_2 (5.2 Ci) trapped on a uranium bed (as uranium tritide) was released by heating (500 °C) and directed in a tritium manifold into a dried reaction vial (332 mbar). After one hour of vigorous stirring the mixture was cooled by liquid nitrogen and the tritium gas was back-trapped on the waste-tritium U-bed. The reaction mixture was filtered through a 0.45 µ PTFE syringe filter and the tritiation flask and filter were washed by three 1 ml portions of DMF. A labile activity was removed by concentration of added CH_3OH/H_2O (50:50) (3 × 2 ml). Afterwards DMF was evaporated on a CentriVap to give crude [^{3}H]-**1** as a white solid. The crude product was dissolved in THF/H_2O (50:50) and 1/5 was used for qualitative and quantitative analysis. Analytical HPLC showed full conversion of starting material and preparative HPLC (method D) gave [^{3}H]-**1** [(95%, 245 nm), 240 mCi, 19.3 Ci/mmol)]. As anticipated ^{1}H NMR showed that the product was a mixture of T and H-reduced products [^{3}H]-**1** and **1** ([^{3}H]-**1**:**1** ratio approx. 5:2 based on the ^{1}H NMR resonance observed at 7.67 ppm).

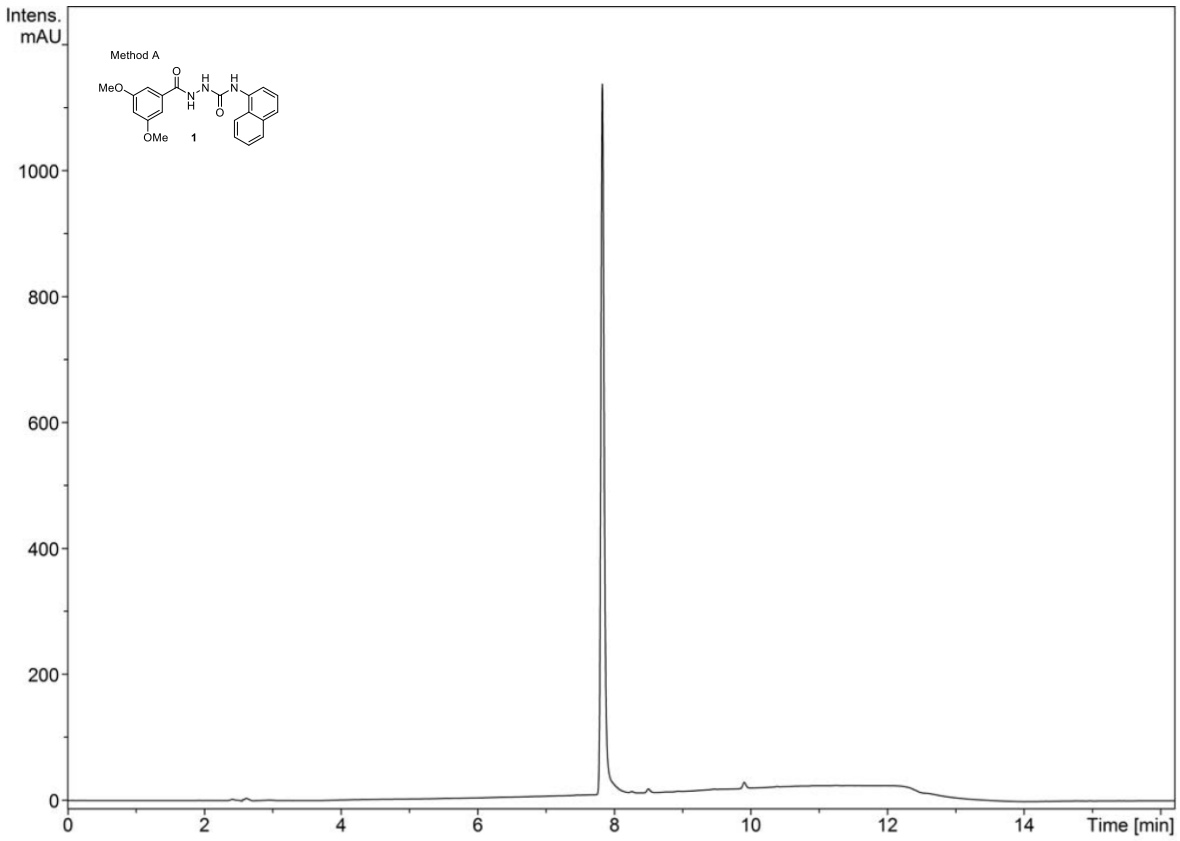
HPLC R_T = 18.3 min (method C), substrate **5** expected at 20.4 min was not detected.

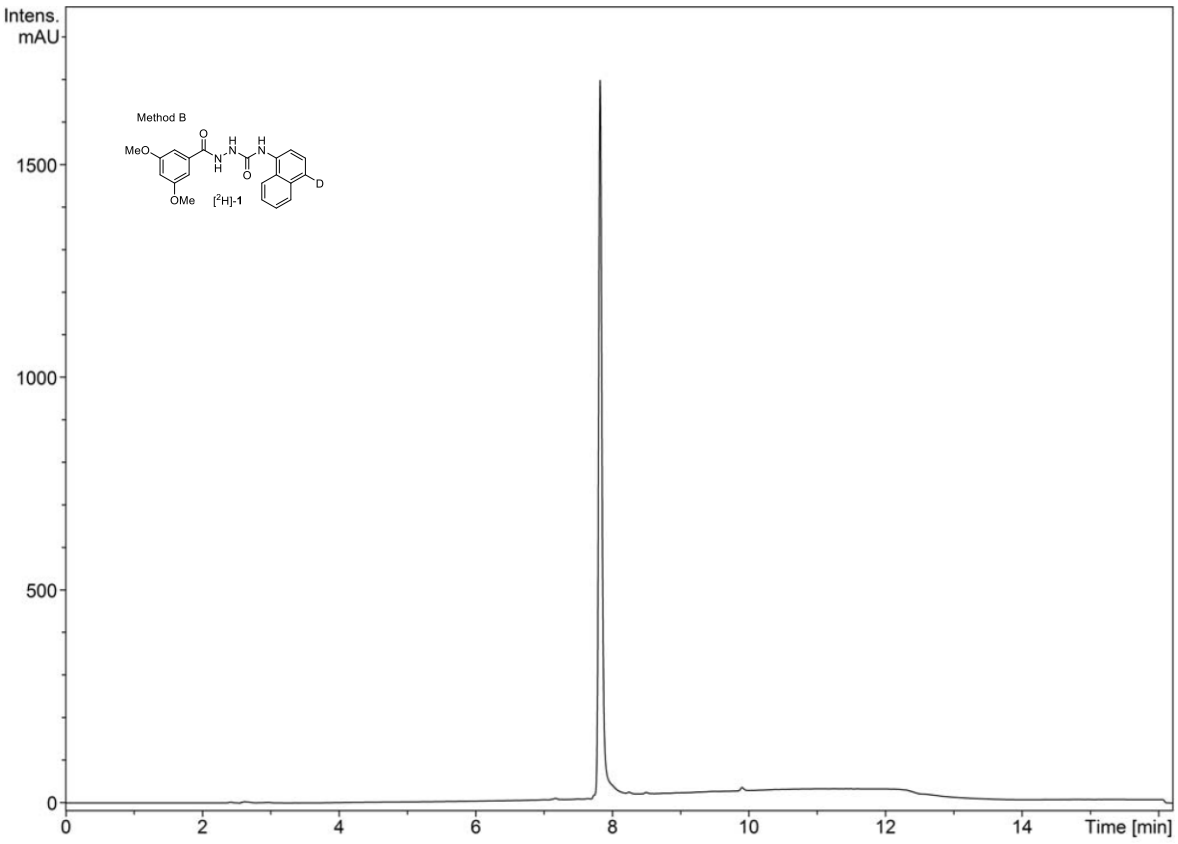
^{1}H NMR (300 MHz, DMSO-*d*6): 10.39 (br s, 1H), 8.94 (s, 1H), 8.47 (s, 1H), 8.09 (d, *J* = 8 Hz, 1H), 7.96 – 7.89 (m, 1H), 7.79 (br s, 1H), 7.61 – 7.50 (m, 2H), 7.47 (t, ^{3}J (^{1}H, ^{3}H) = 8.5 Hz, 1H), 7.10 (d, *J* = 2.5 Hz, 2H), 6.70 (t, *J* = 2.5 Hz, 1H), 3.80 (s, 6H).

^{3}H-^{1}H decoupled NMR (320 MHz, DMSO-*d*6): δ 7.66 (s).

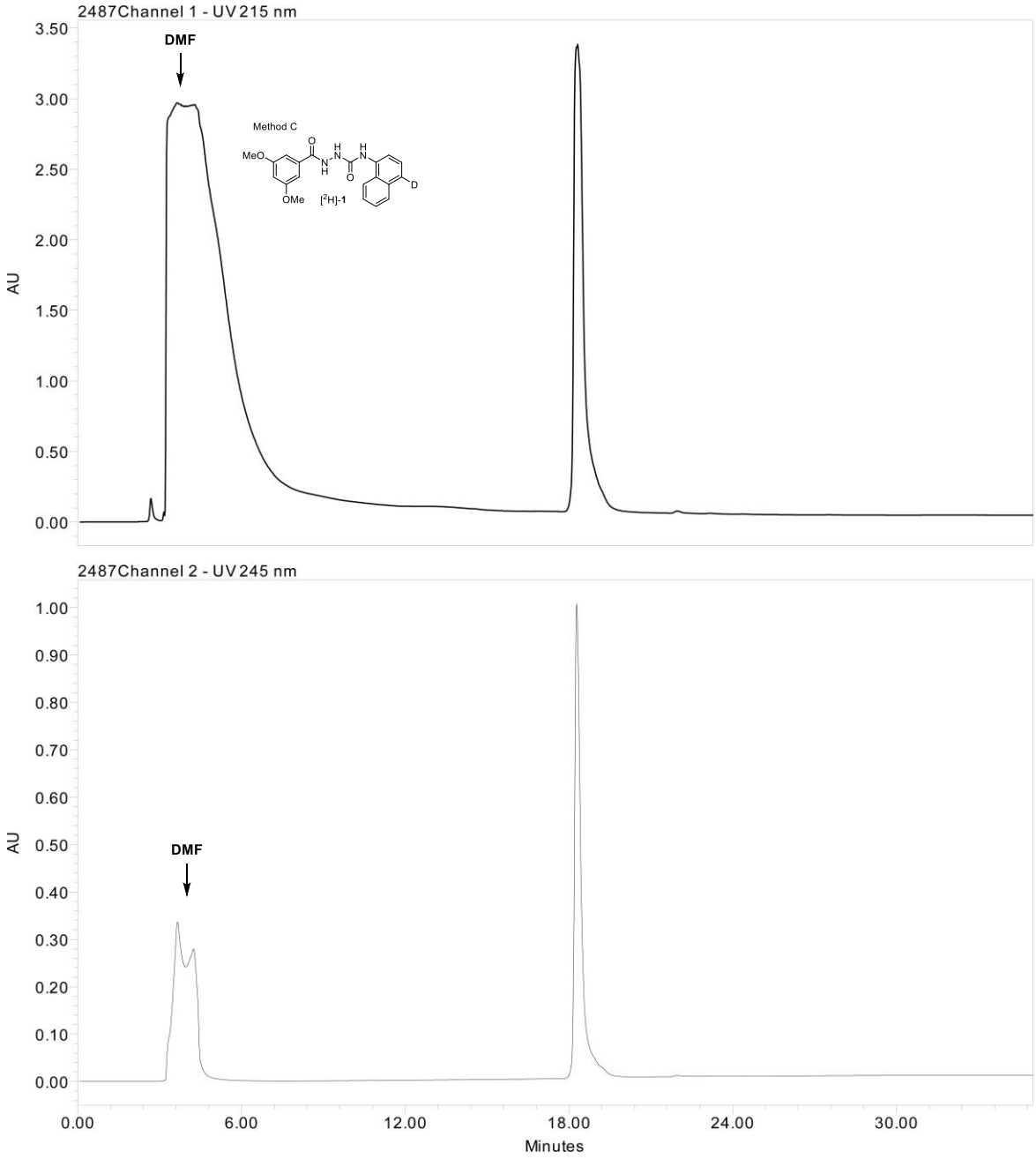
^{3}H-^{1}H coupled NMR (320 MHz, DMSO-*d*6): δ 7.66 (d, *J* = 6.5 Hz).

HPLC CHROMATOGRAMS

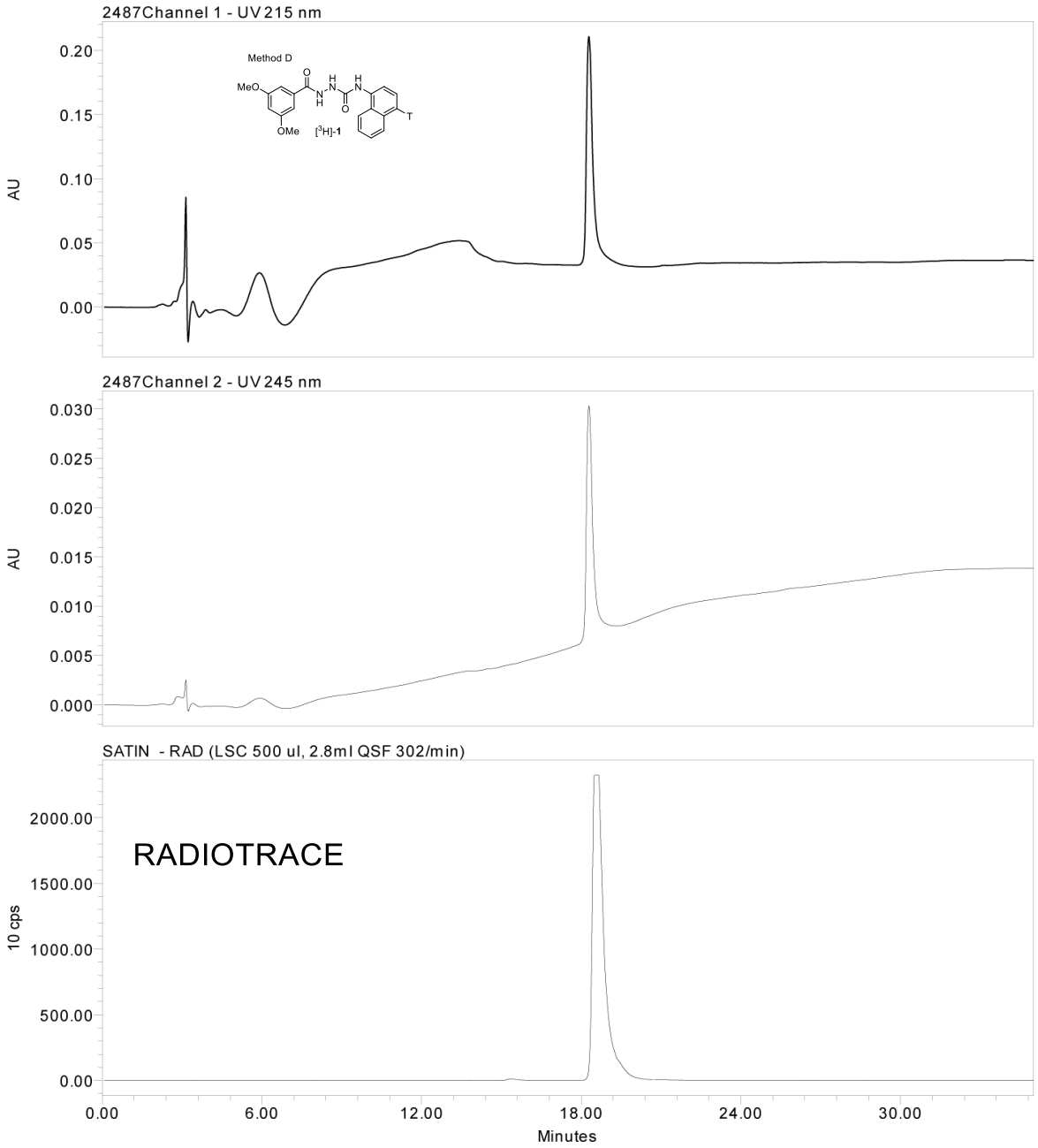


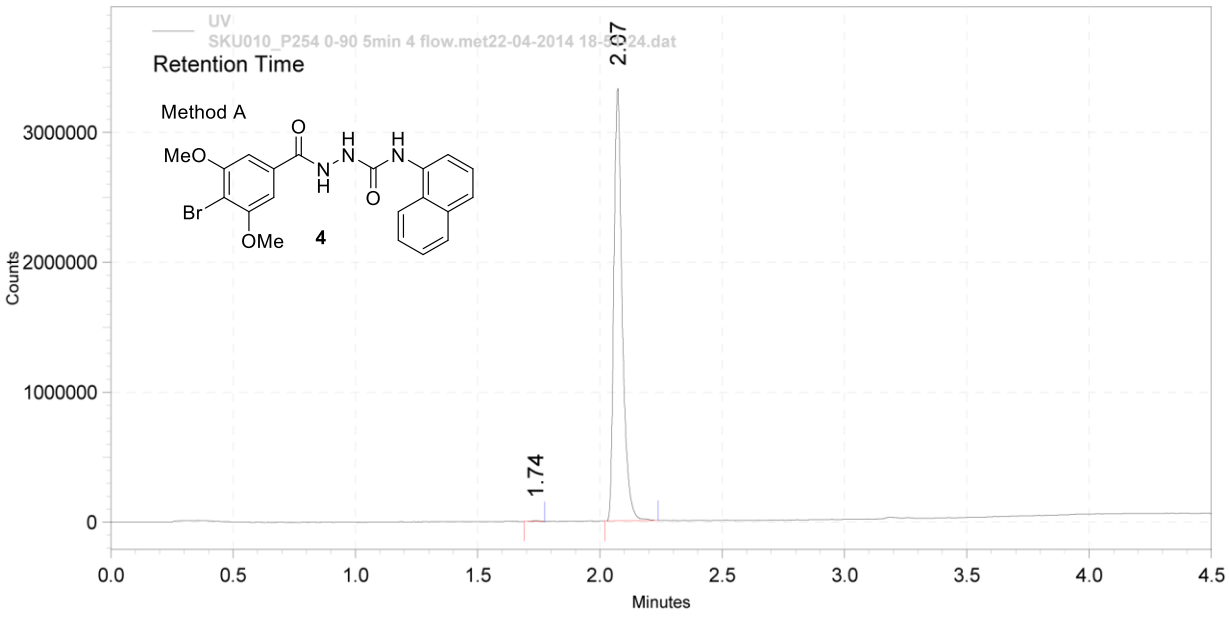

Intens.
mAU
1500
1000
500
0
0
2
4
6
8
10
12
14
Time [min]
Method B
MeO
OMe
[2H]-1
D

Optimised deuteration conditions



HPLC chromatograms and radiotrace.




UV
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Retention Time
Method A
MeO
Br
OMe
4
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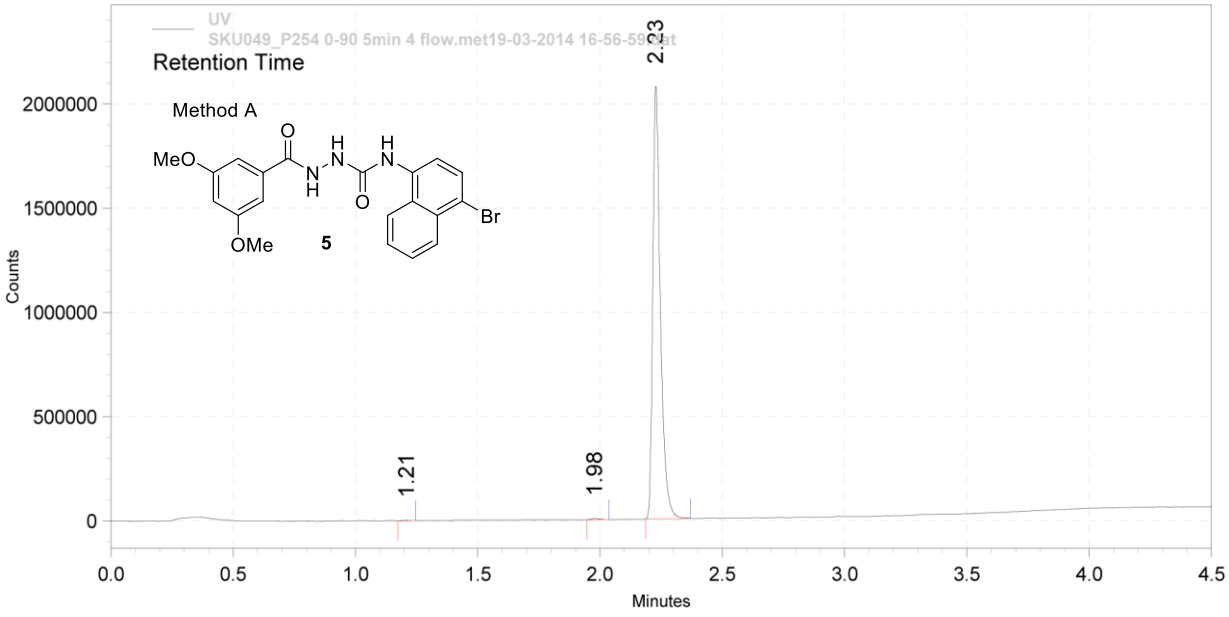
1.74
Counts
3000000
2000000
1000000
0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
Minutes


UV
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Retention Time
Method A
MeO
OMe
Br
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2.23
1.98
1.21
Counts
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1500000
1000000
500000
0
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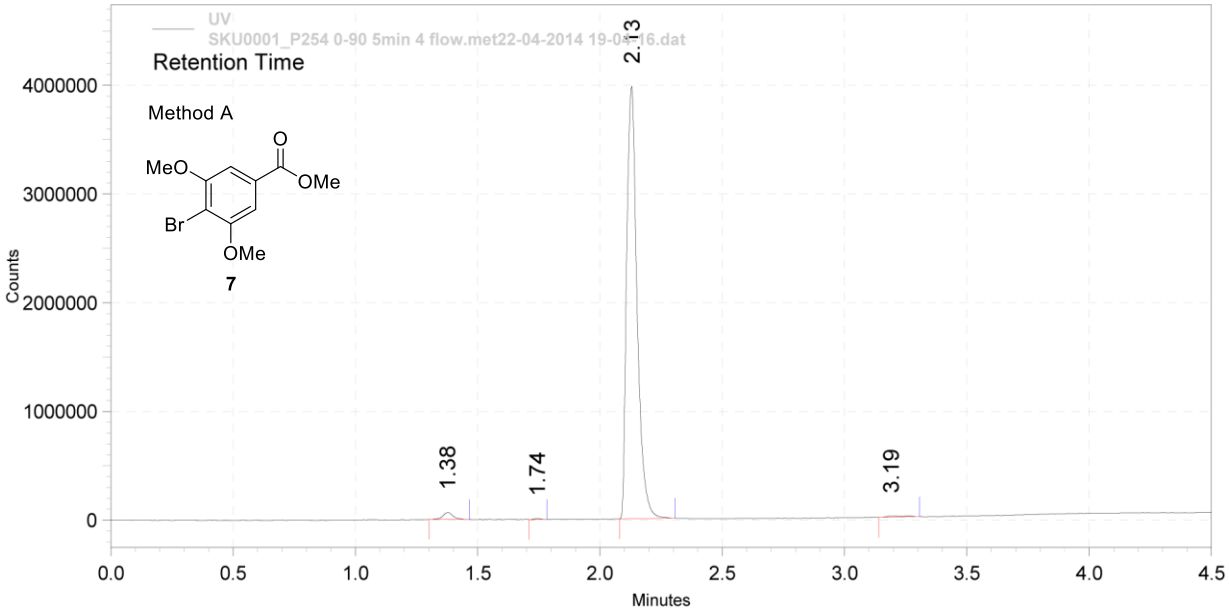
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
Minutes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Retention Time
Method A
MeO
OMe
Br
OMe
7
Counts
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3000000
2000000
1000000
0
1.38
1.74
2.13
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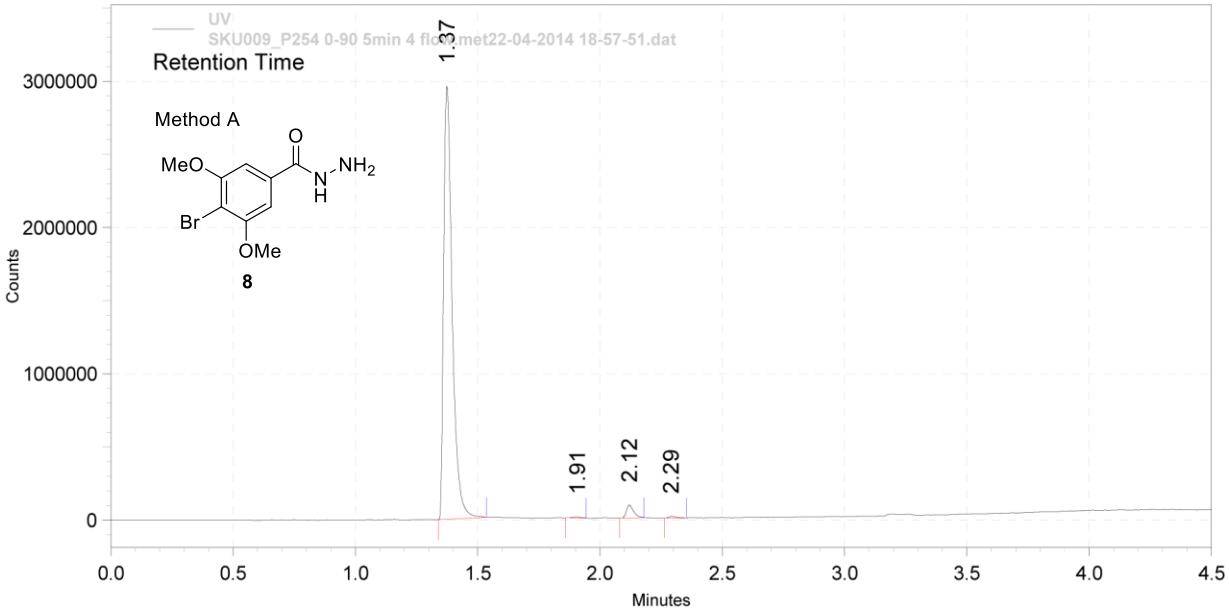
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
Minutes


UV
SKU009_P254 0-90 5min 4 flo[illegible]met22-04-2014 18-57-51.dat
Retention Time
Method A
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NH2
N
H
Br
OMe
8
Counts
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2000000
1000000
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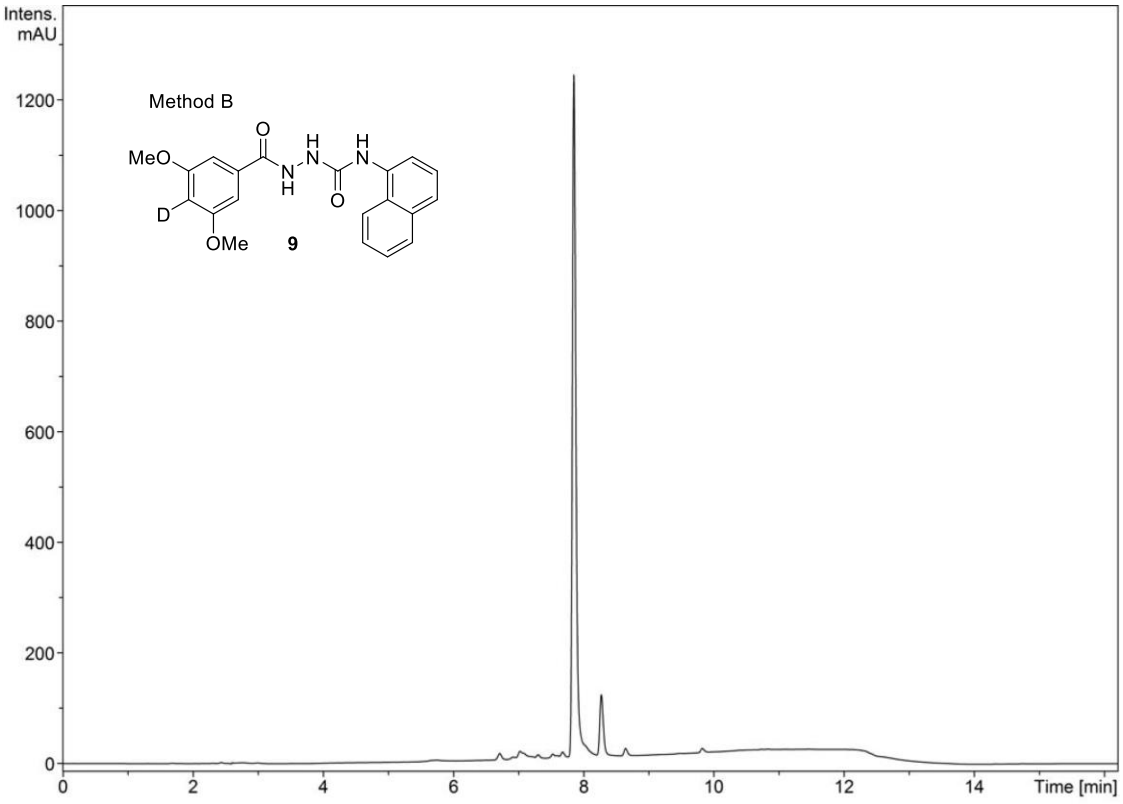
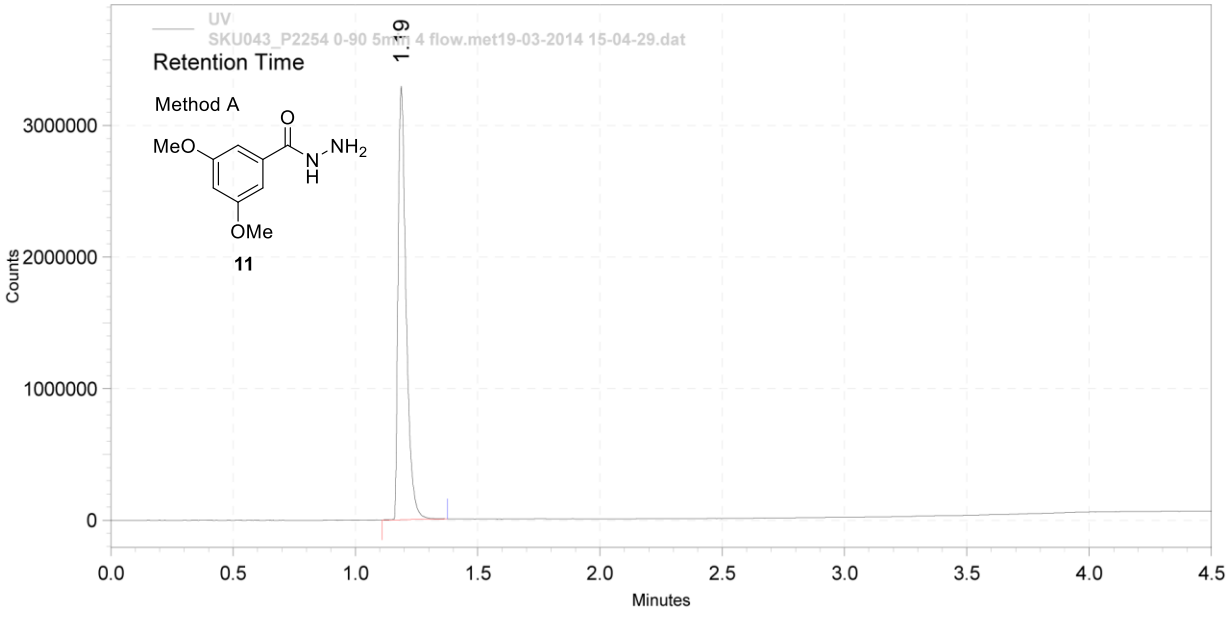
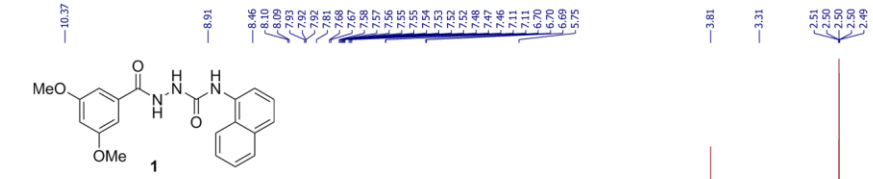
3.0
3.5
4.0
4.5
Minutes

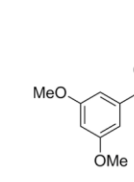

Intens.
mAU
1200
1000
800
600
400
200
0
Method B
MeO
D
OMe
9
0
2
4
6
8
10
12
14
Time [min]


UV
SKU043_P2254 0-90 5mM 4 flow.met19-03-2014 15-04-29.dat
Retention Time
1.19
Method A
MeO
NH2
OMe
11
Counts
3000000
2000000
1000000
0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
Minutes

^{1}H, ^{3}H AND ^{13}C NMR SPECTRA

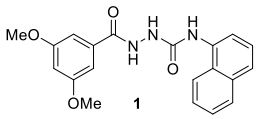


600 MHz, ^{1}H NMR, DMSO-*d6*



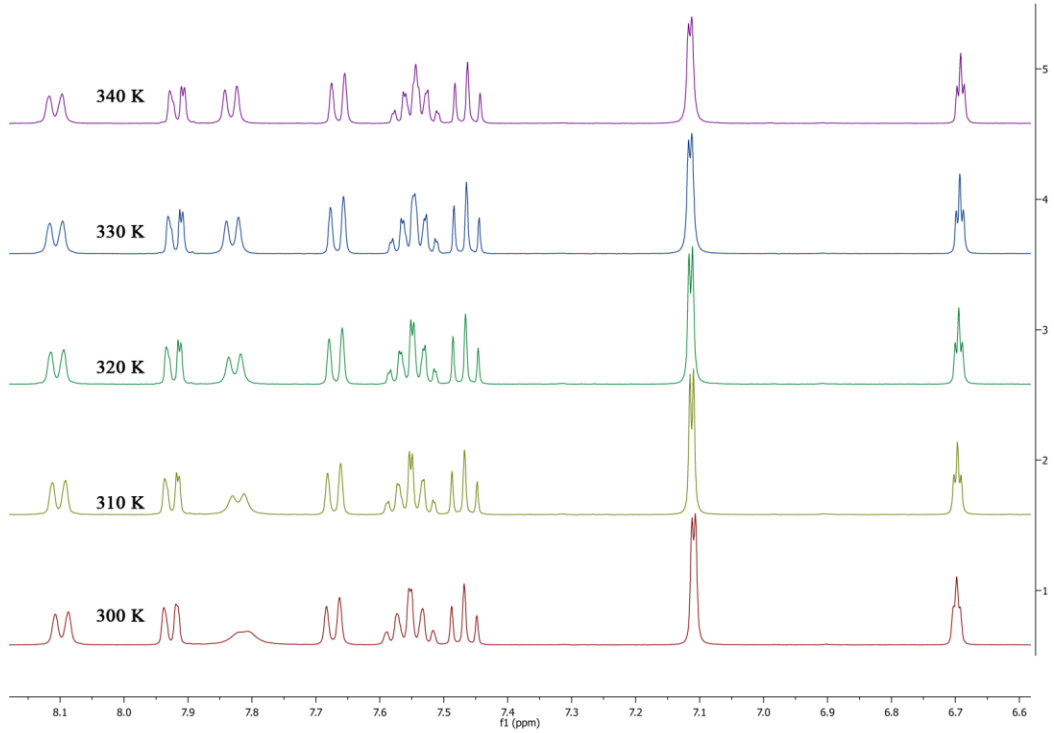
151 MHz, ^{13}C NMR, DMSO-*d6*

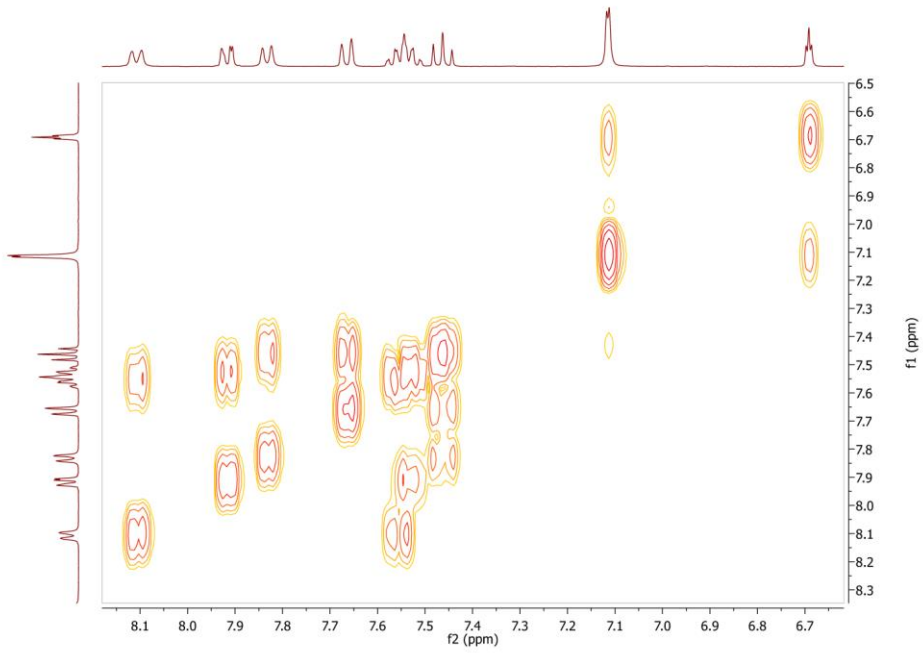
High temperature ^{1}H and COSY (340 K) NMR of **1**, zoom in aromatic region



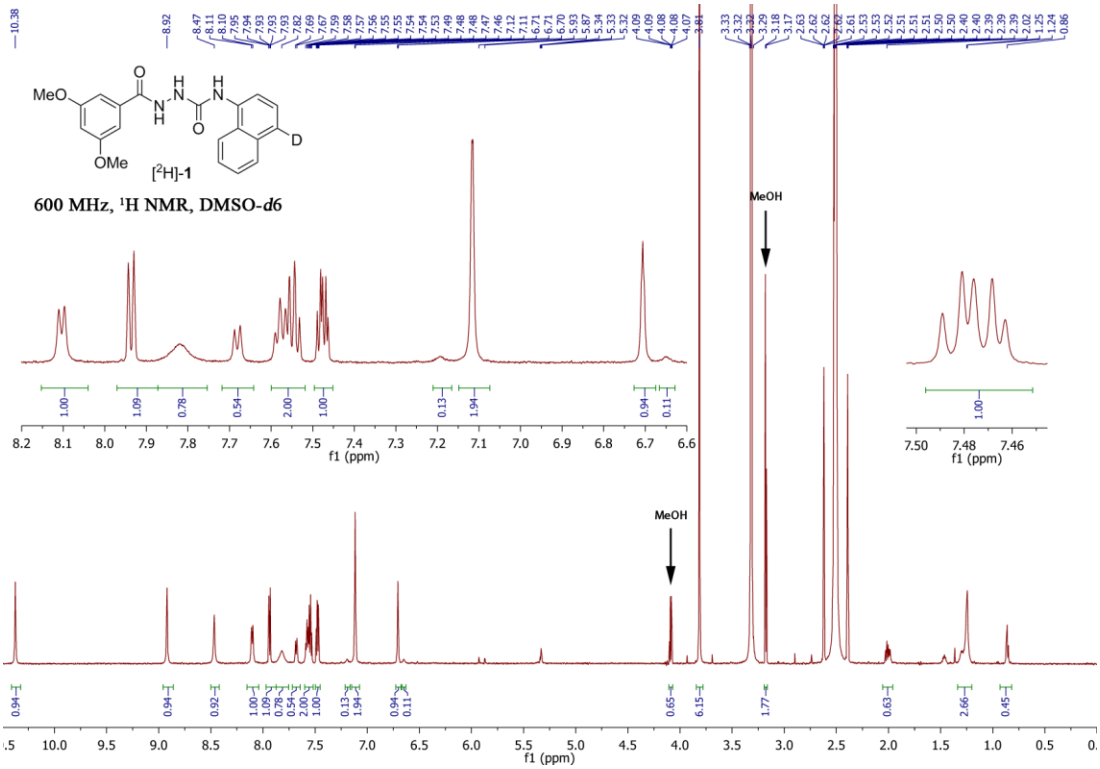
1

400 MHz, ^{1}H NMR, DMSO-*d6*



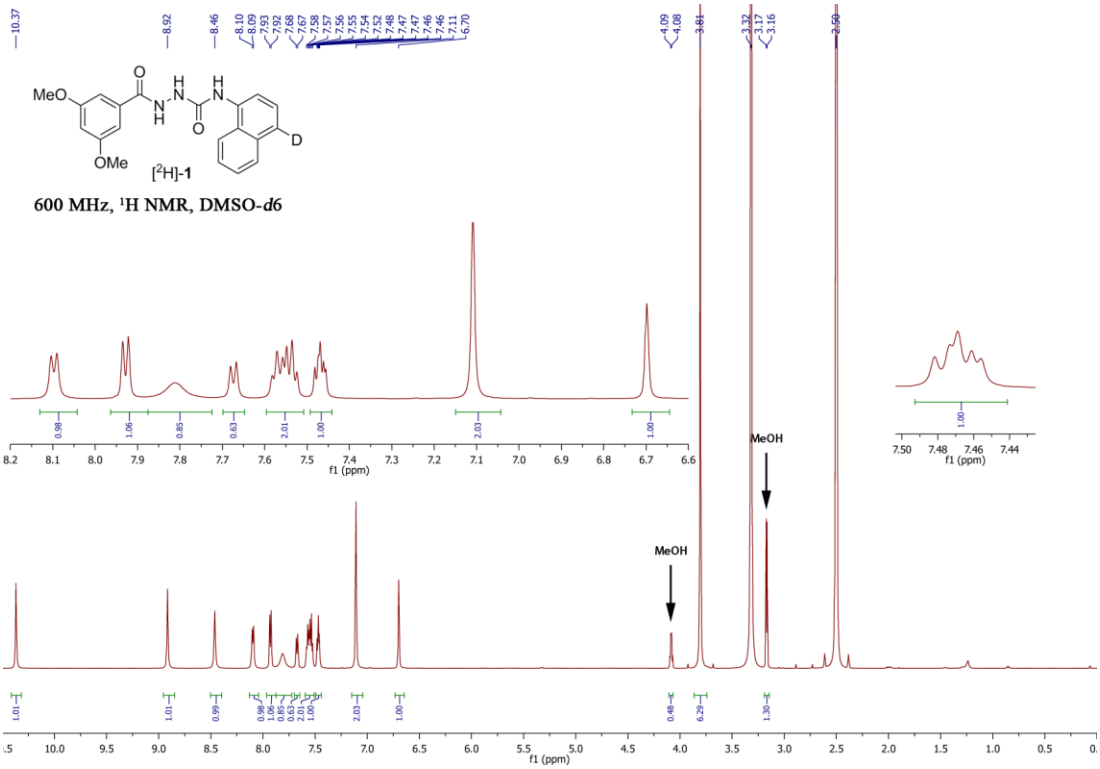


Deuteration with precursor **5** (30 mg scale reaction)



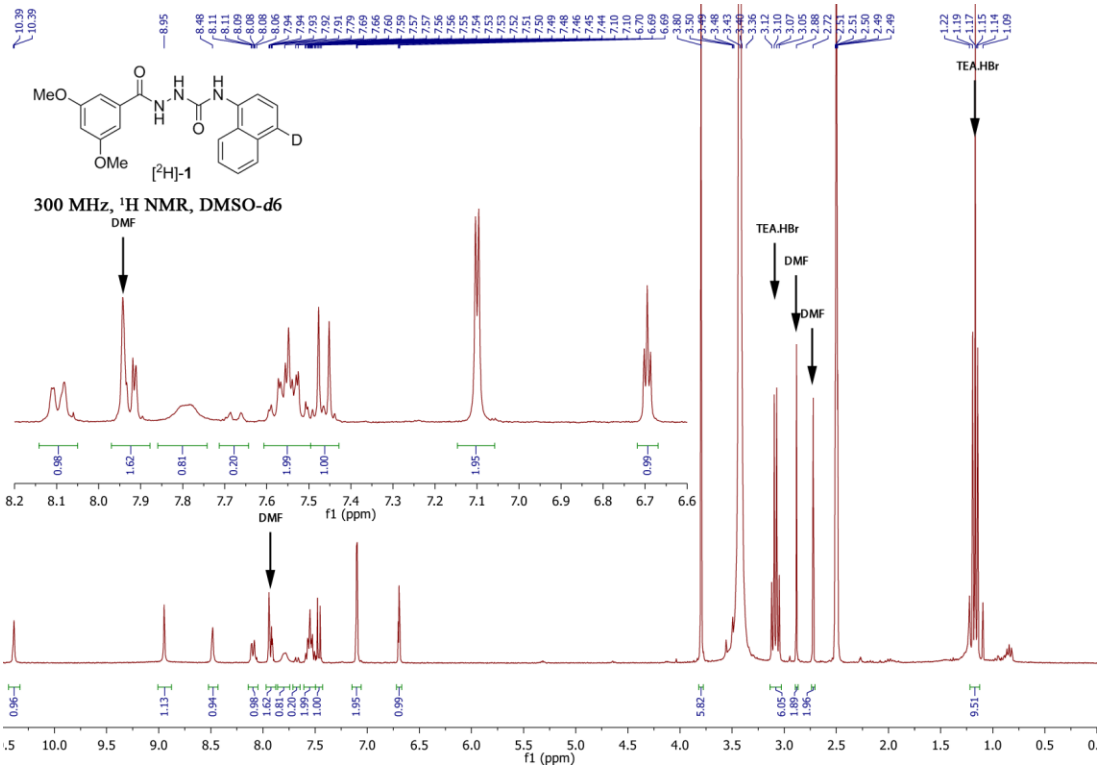
Mixture of D and H-reduced products [^{2}H]-**1** and **1**. The ratio is approximately 1:1 based on the ^{1}H NMR resonance observed at 7.67 ppm.

Deuteration with precursor **5** (100 mg scale reaction)



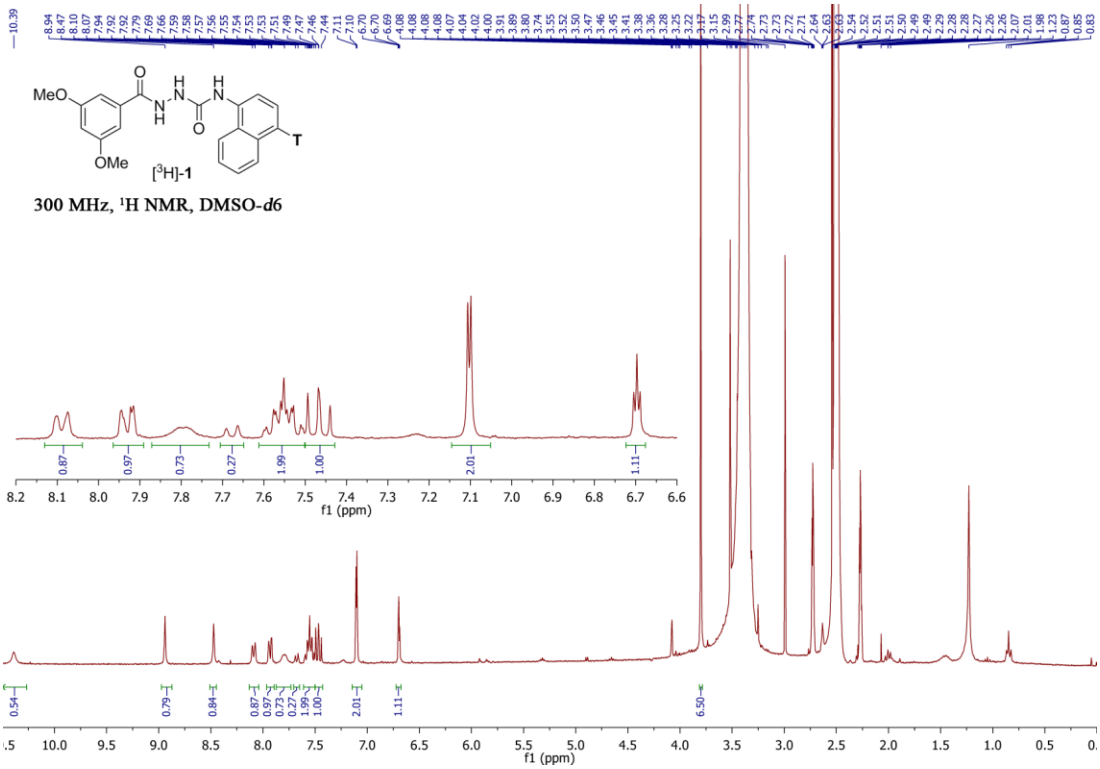
Mixture of D and H-reduced products [^{2}H]-**1** and **1**. The ratio is approximately 2:3 based on the ^{1}H NMR resonance observed at 7.67 ppm.

Deuteration with precursor **5**: optimised conditions

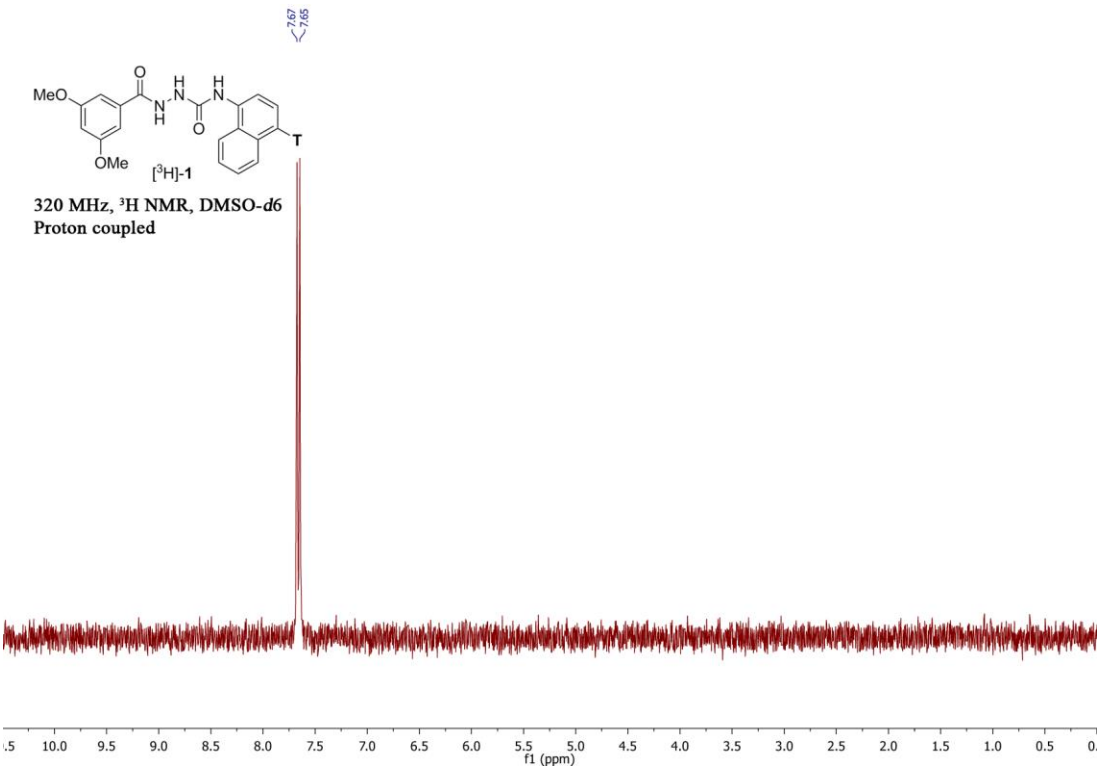


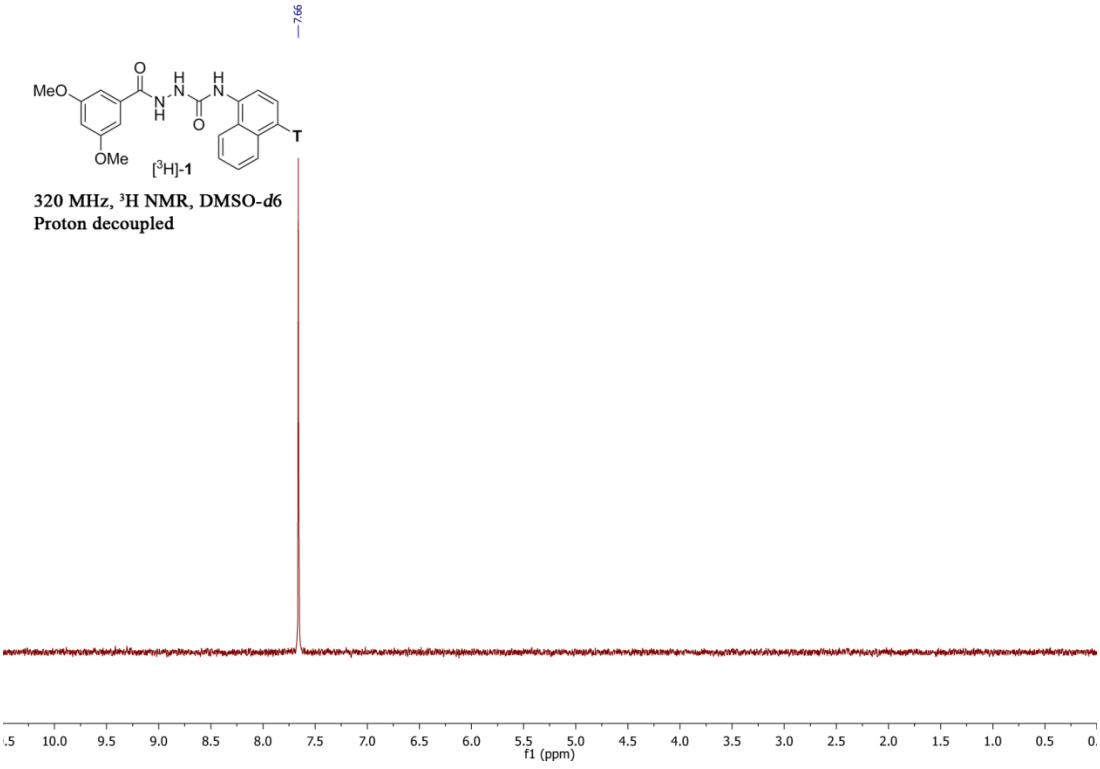
Mixture of D and H-reduced products [^{2}H]-**1** and **1**. The ratio is approximately 4:1 based on the ^{1}H NMR resonance observed at 7.67 ppm.

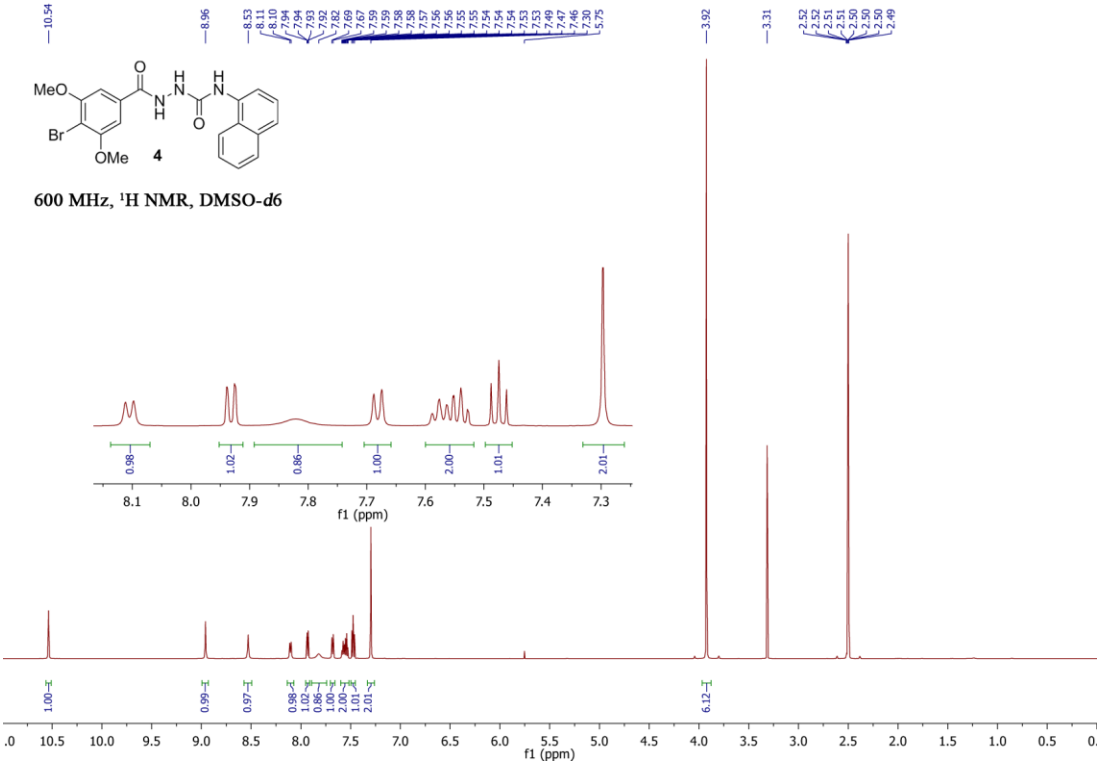
Tritiation with precursor **5**

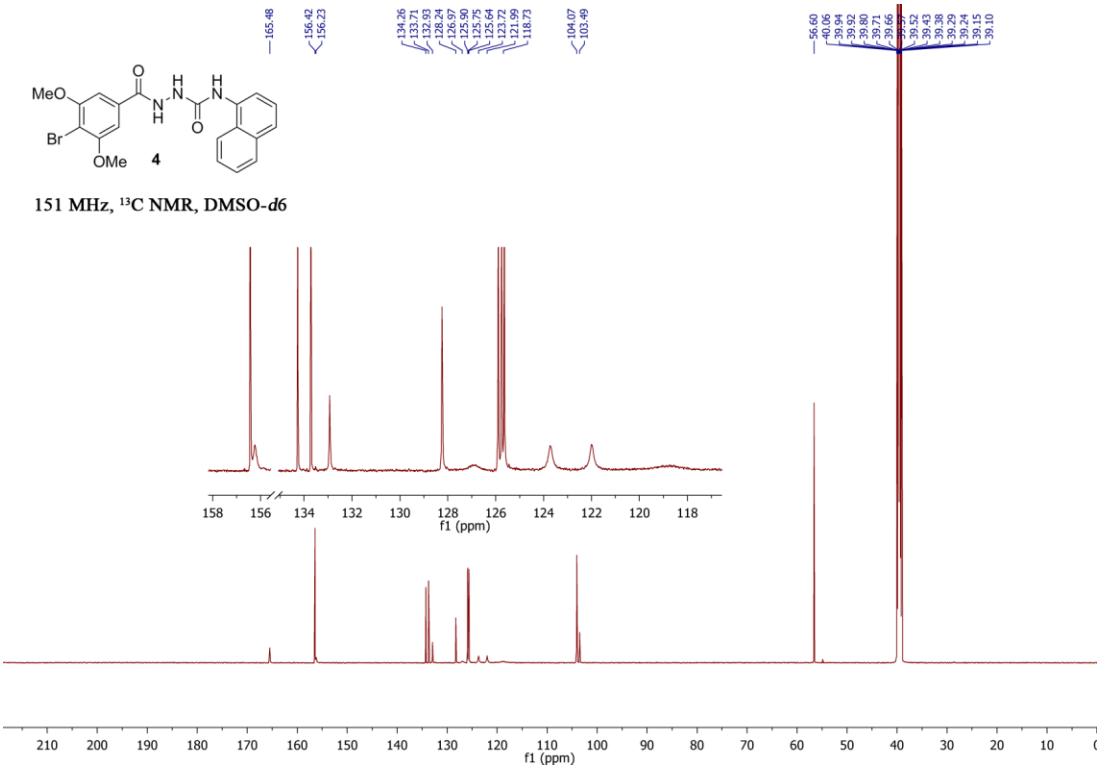


Mixture of T and H-reduced products [^{3}H]-**1** and **1**. The ratio is approximately 5:2 based on the ^{1}H NMR resonance observed at 7.67 ppm.

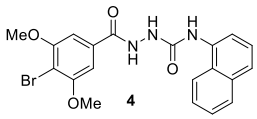

7.67
7.65
MeO
OMe
T
[3H]-1
320 MHz, 3H NMR, DMSO-d6
Proton coupled
10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5
f1 (ppm)


7.66
MeO
OMe
T
[3H]-1
320 MHz, 3H NMR, DMSO-d6
Proton decoupled
10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5
f1 (ppm)


MeO
Br
OMe
4
600 MHz, ¹H NMR, DMSO-d6
f1 (ppm)

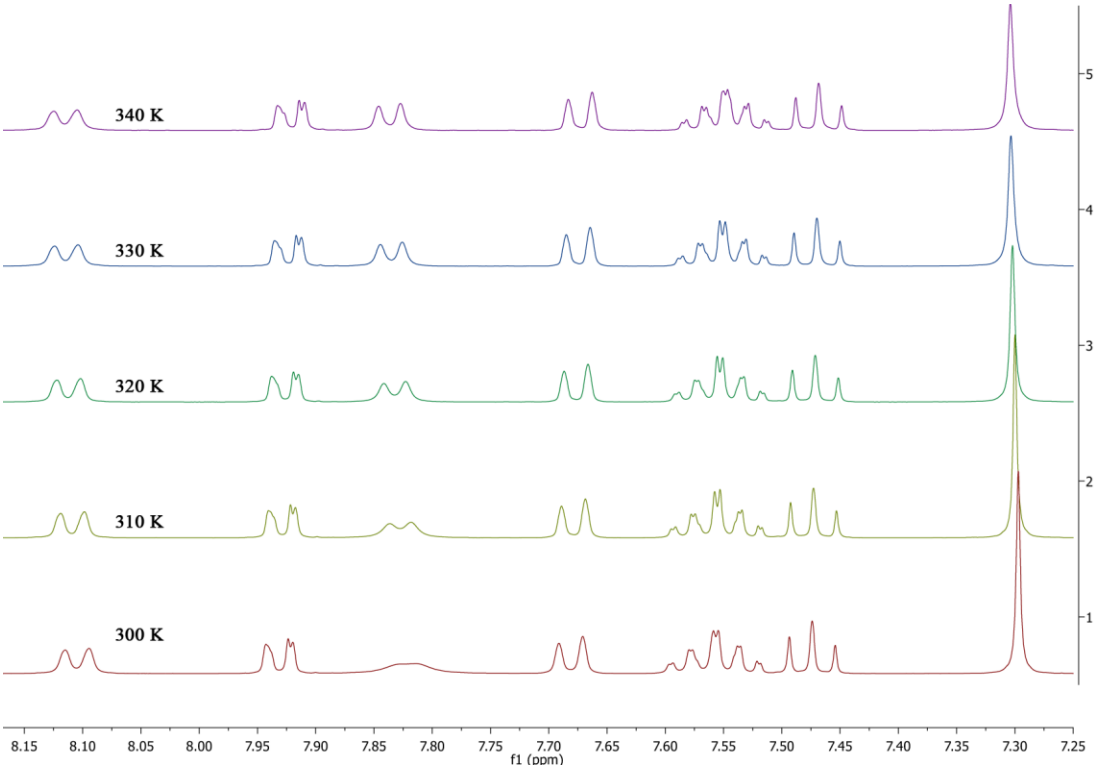

MeO
Br
OMe
4
151 MHz, ¹³C NMR, DMSO-d6
f1 (ppm)

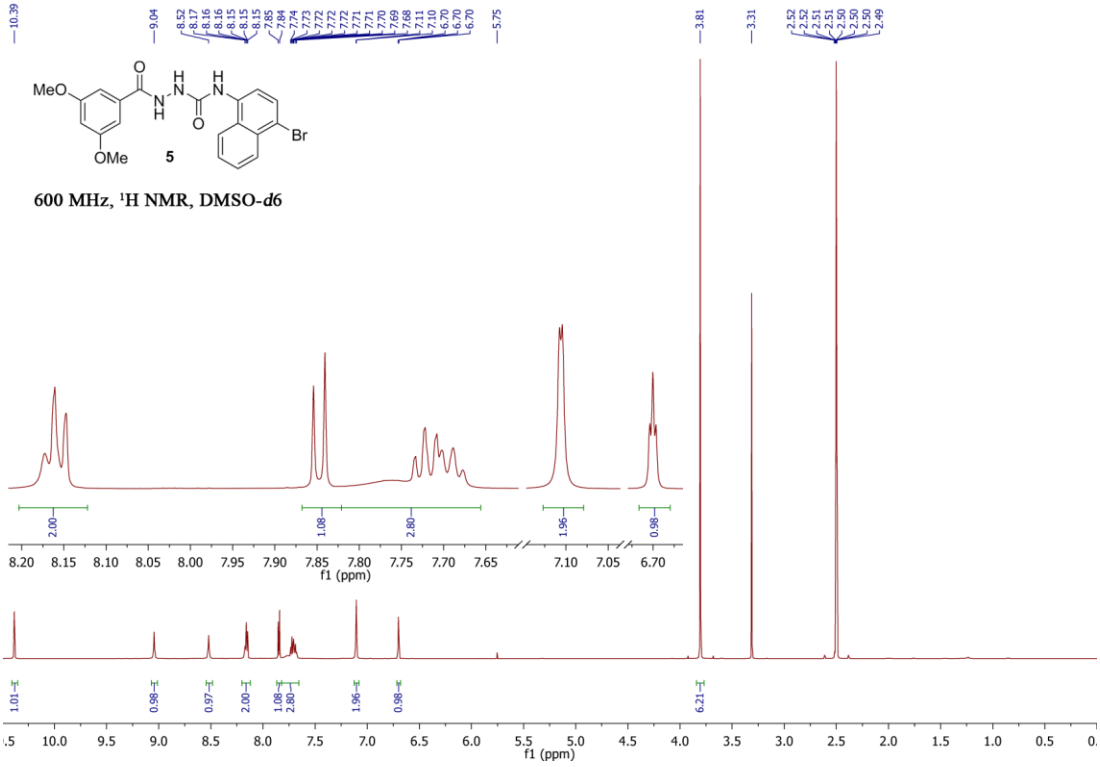
High temperature ^{1}H NMR of **4**, zoom in aromatic region



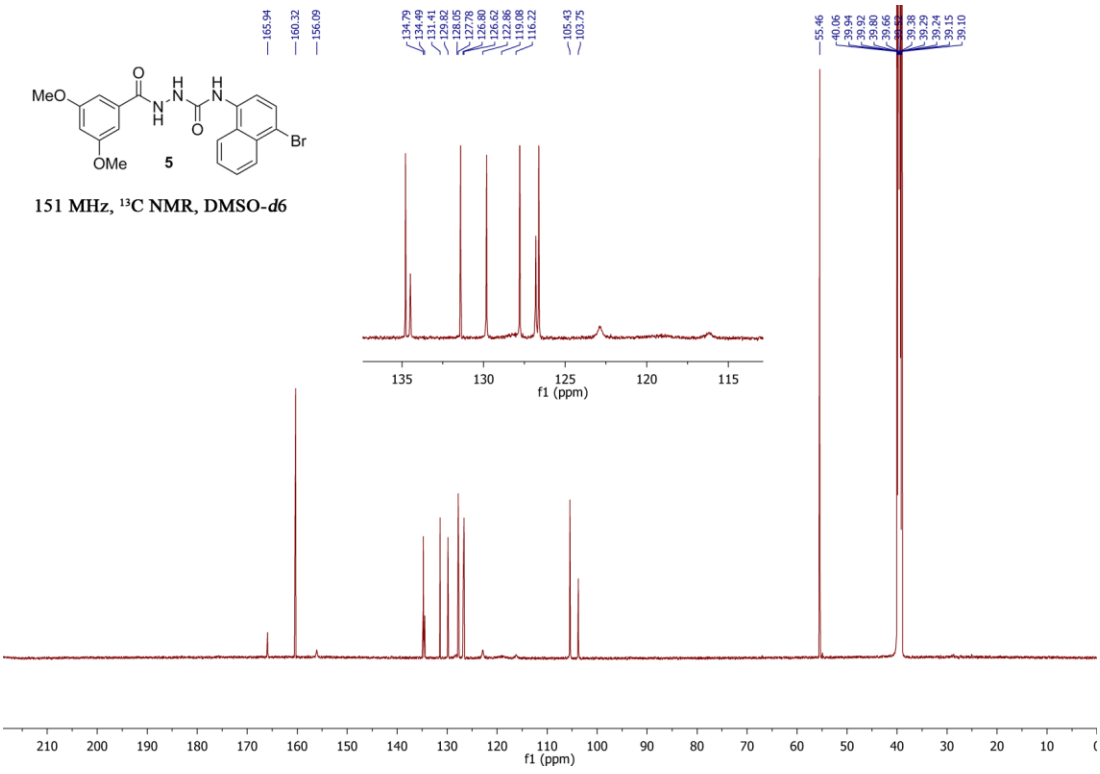
4

400 MHz, ^{1}H NMR, DMSO-*d6*



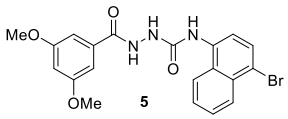


600 MHz, ^{1}H NMR, DMSO-*d6*



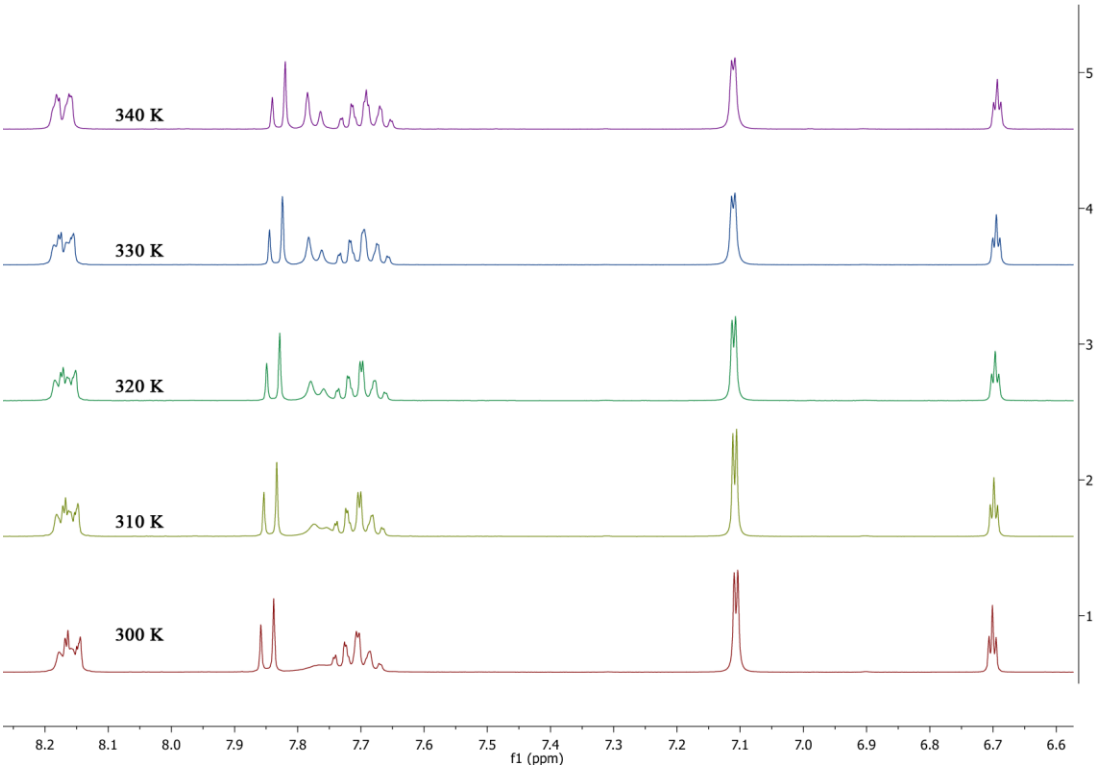
151 MHz, ^{13}C NMR, DMSO-*d6*

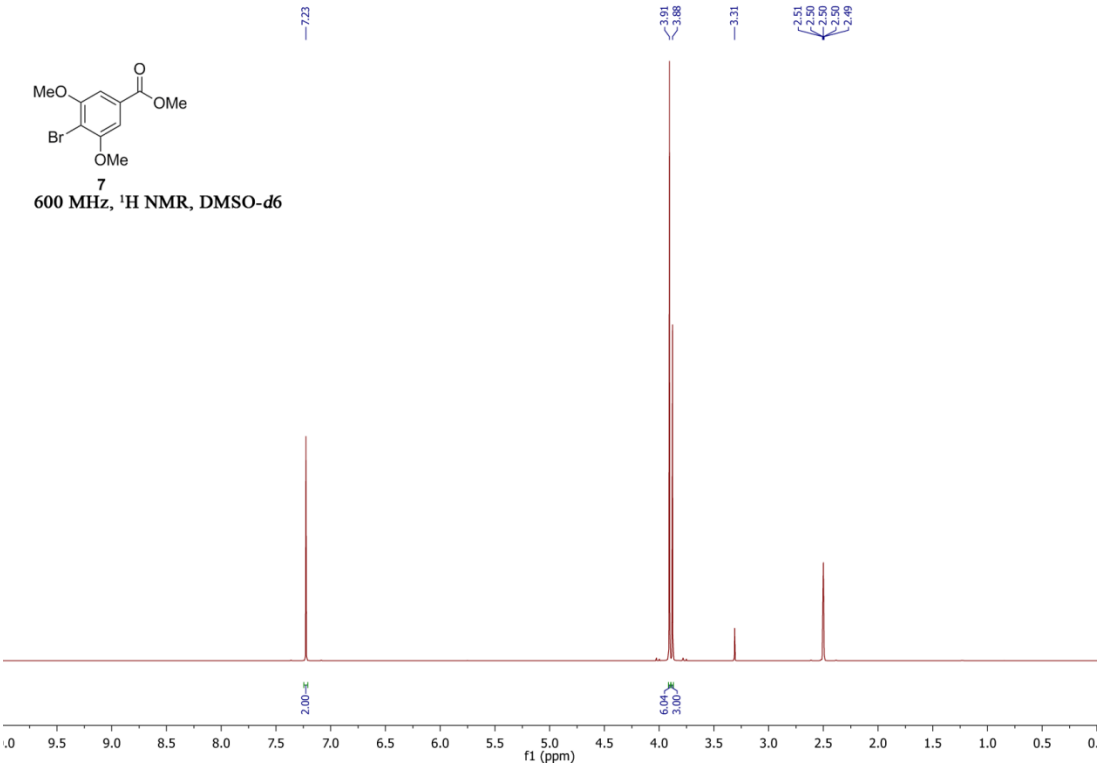
High temperature ^{1}H NMR of **5**, zoom in aromatic region



5

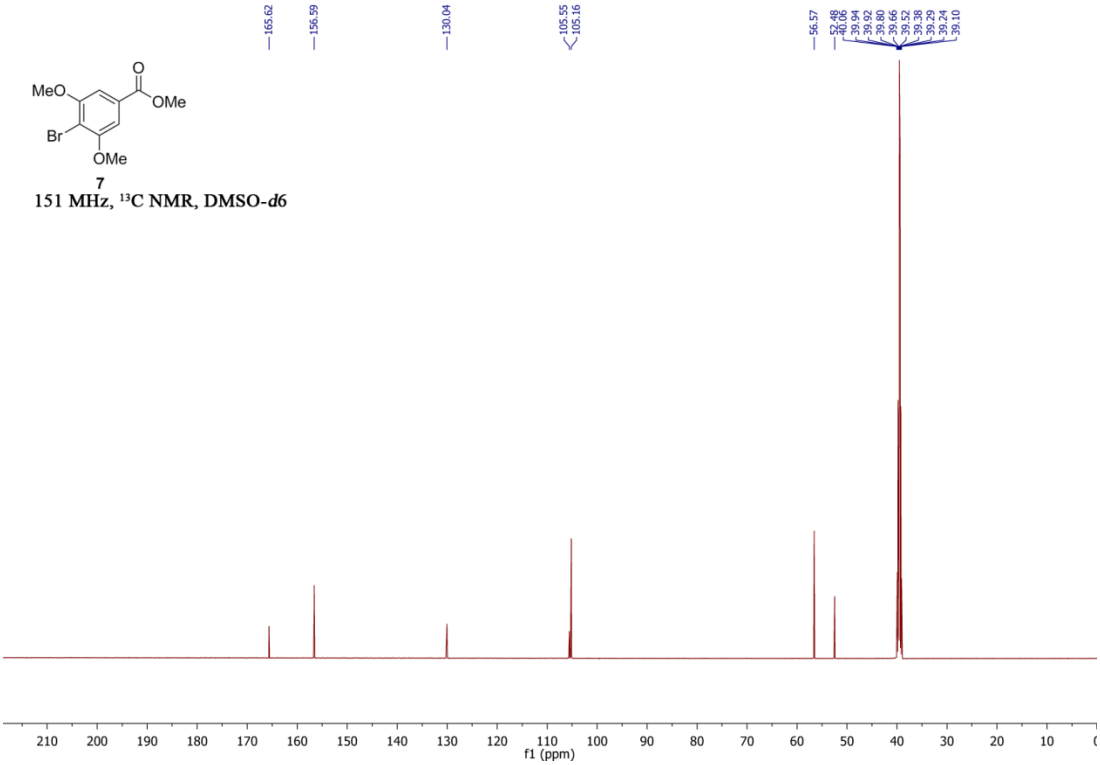
400 MHz, ^{1}H NMR, DMSO-*d6*





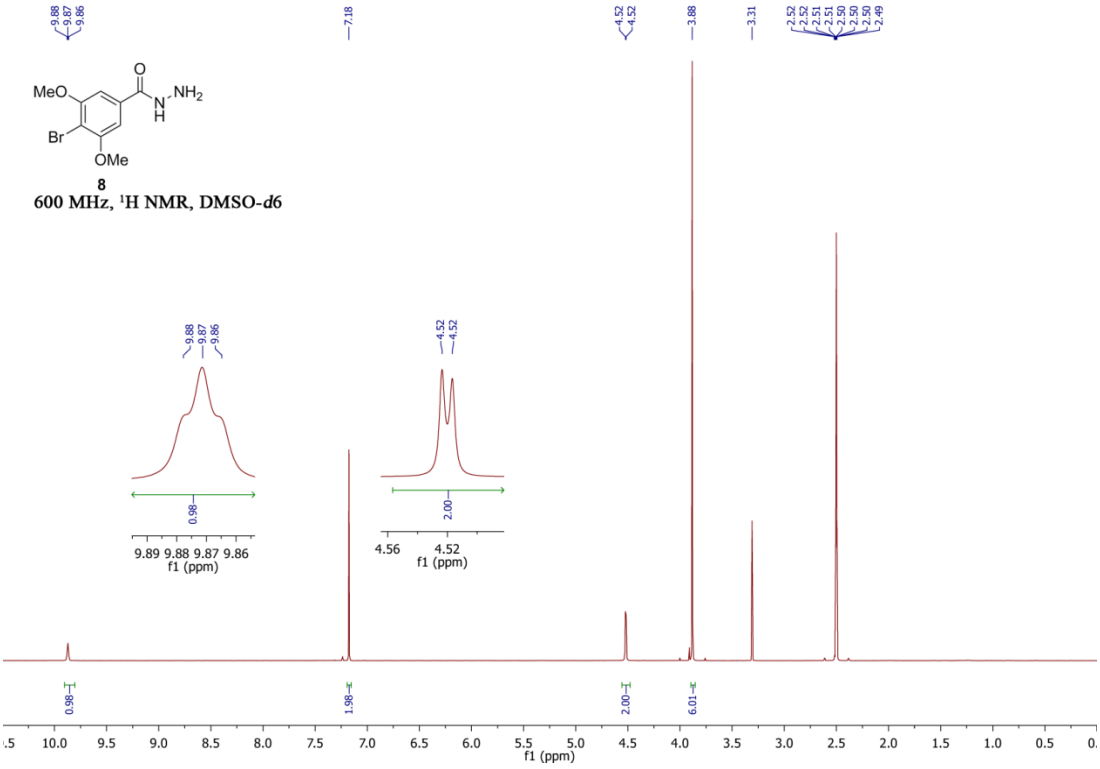
7

600 MHz, ^{1}H NMR, DMSO-*d6*



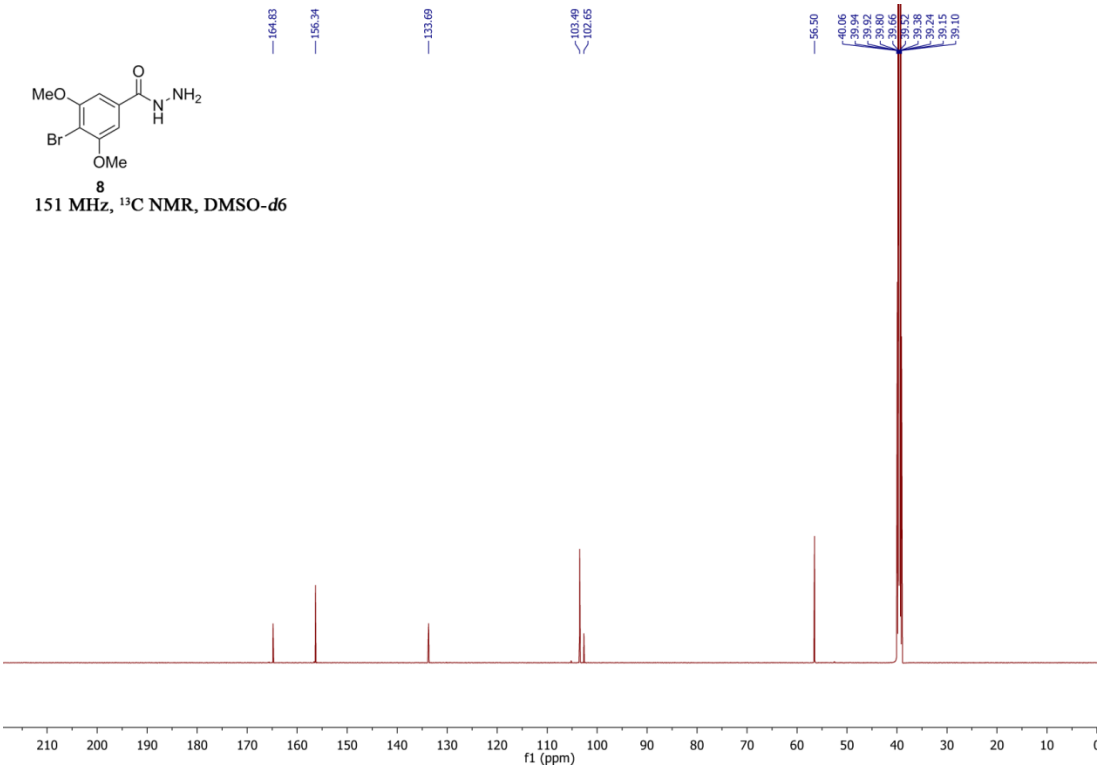
7

151 MHz, ^{13}C NMR, DMSO-*d6*



8

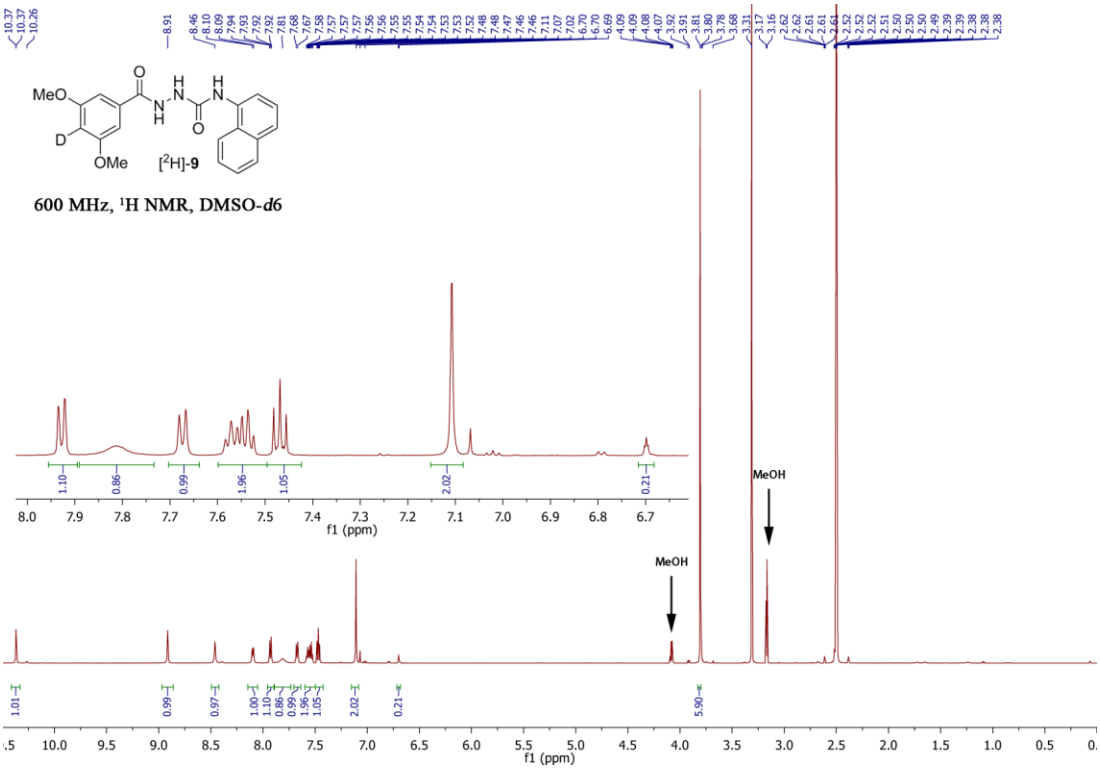
600 MHz, ^{1}H NMR, DMSO-*d6*



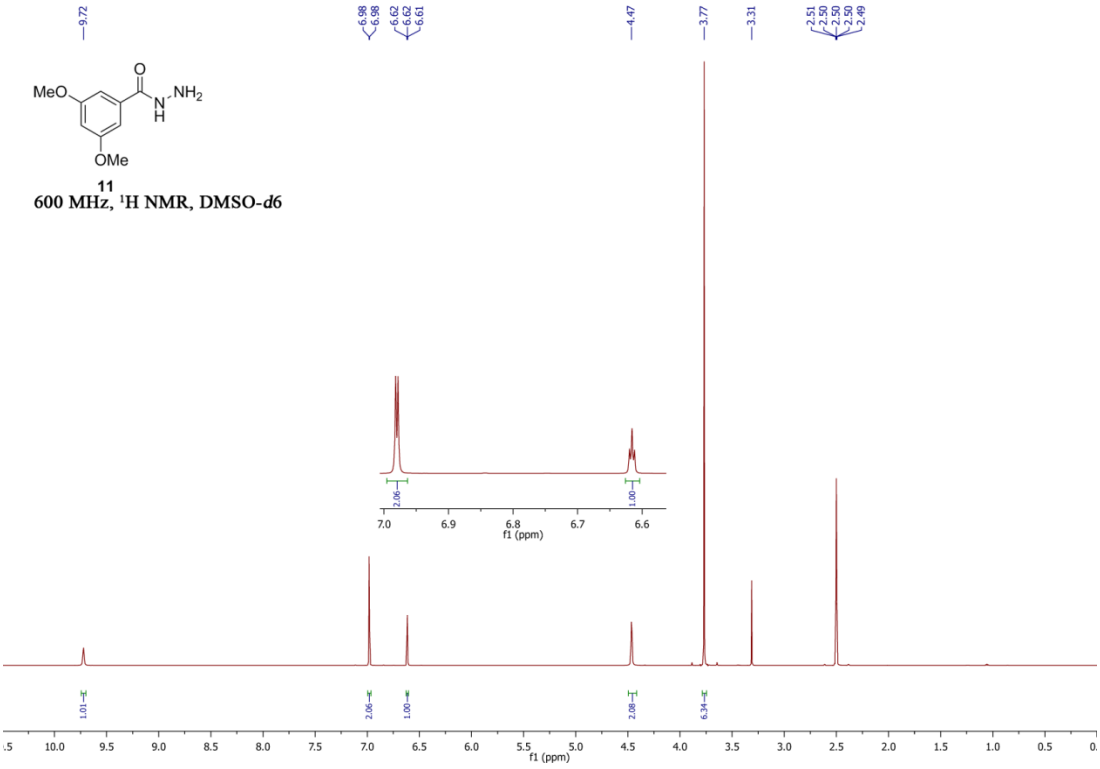
8

151 MHz, ^{13}C NMR, DMSO-*d6*

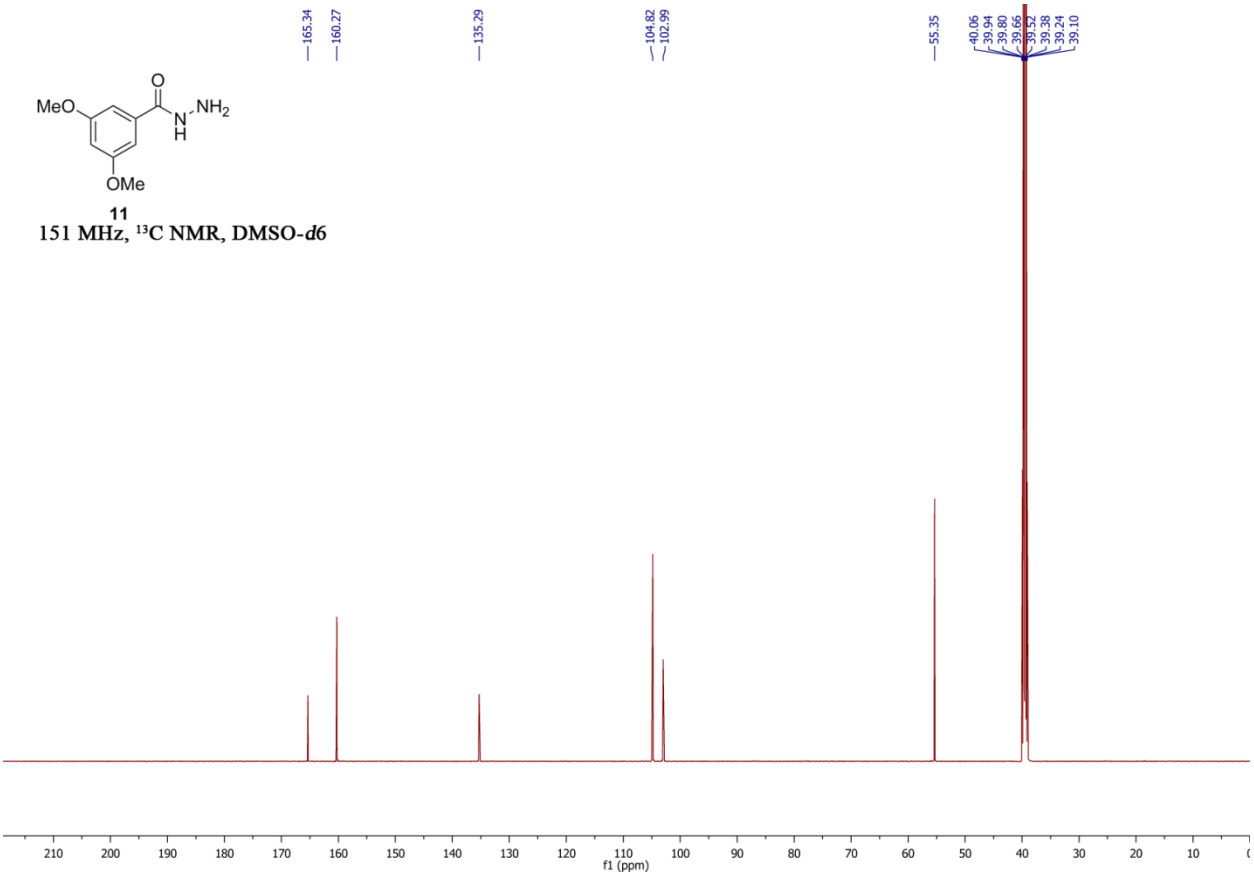
Deuteration with precursor **4** (30 mg scale reaction)



Mixture of D and H-reduced products [^{2}H]-**9** and **1**. The ratio is approximately 4:1 based on the ^{1}H NMR resonance observed at 6.70 ppm.

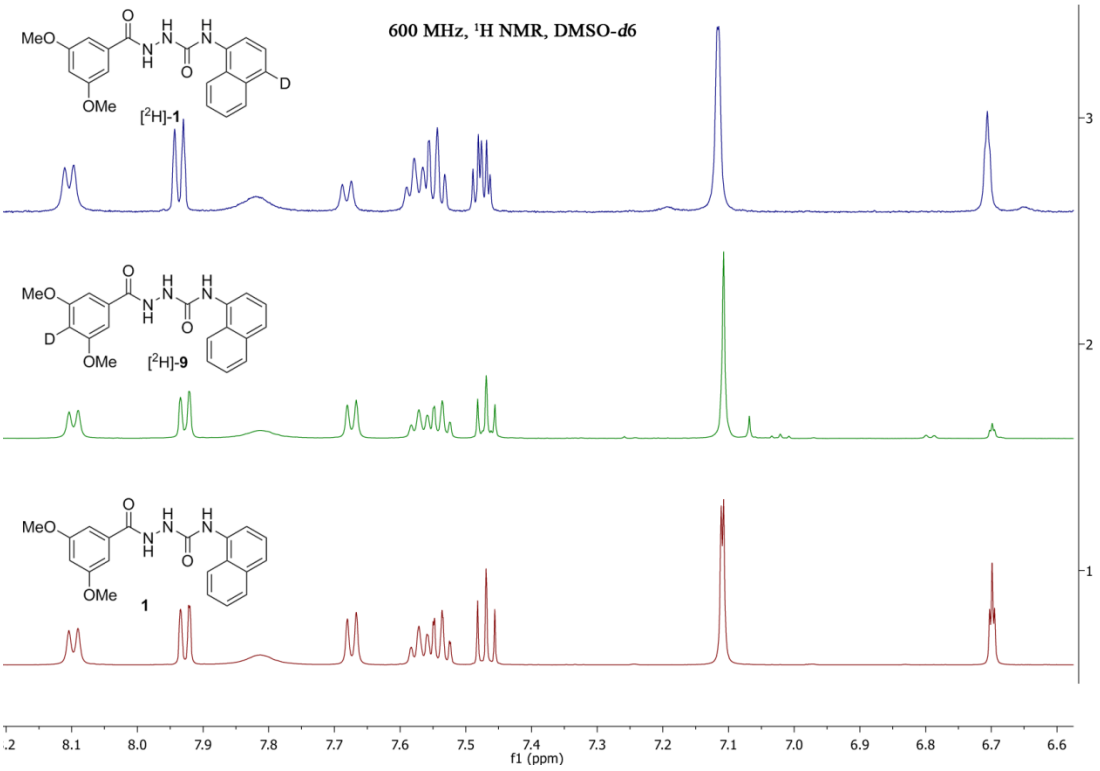


11
600 MHz, ^{1}H NMR, DMSO-*d6*



11
151 MHz, ^{13}C NMR, DMSO-*d6*

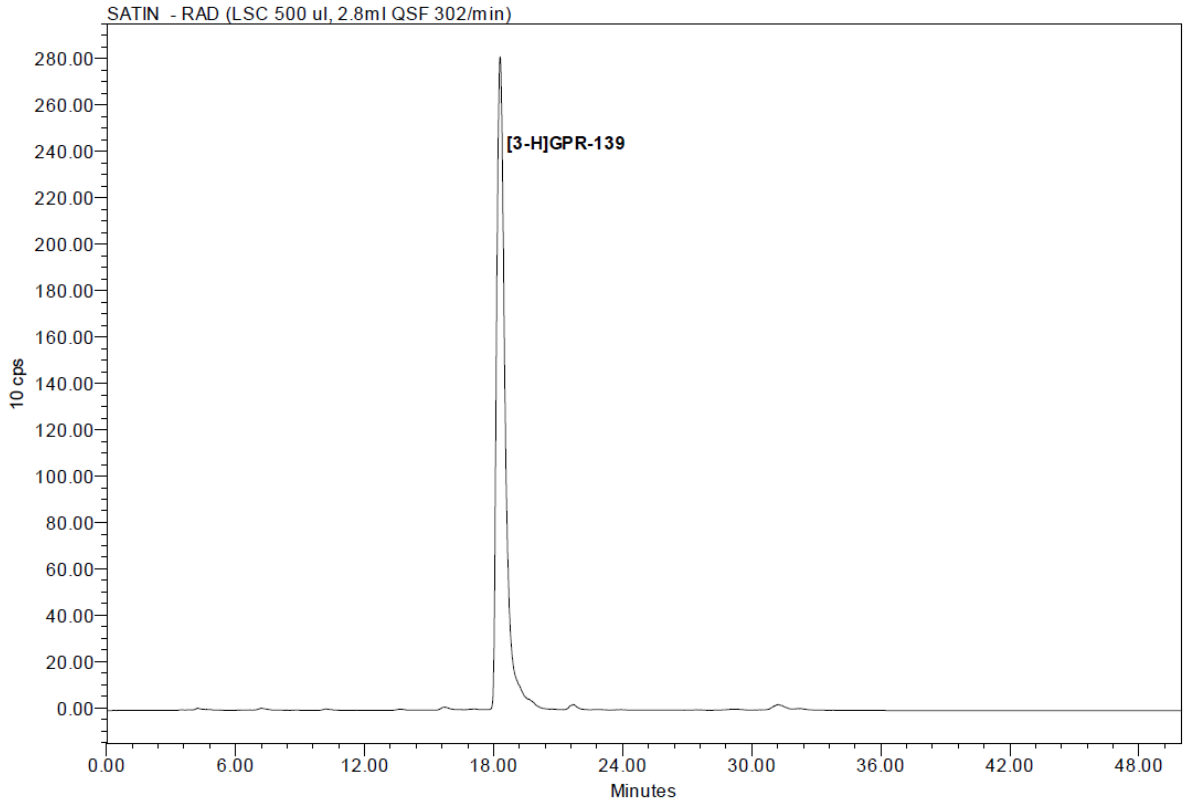
Stacked spectra of **1**, **9** and [^{2}H]-**1**, zoom in aromatic region



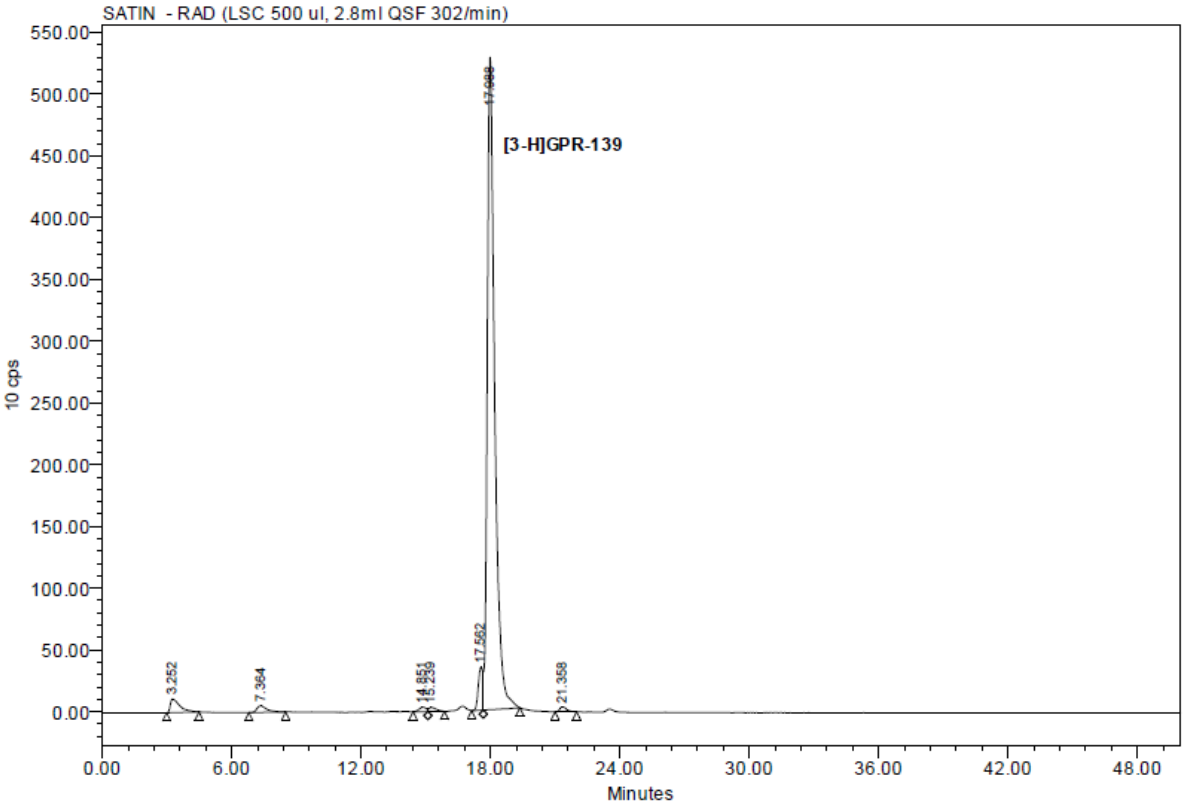
RADIOSTABILITY OF [^{3}H]-1

Stability studies were performed in order to determine the best formulation for storage and use in pharmacological investigations of the radioligand. The rate of decomposition of [^{3}H]-**1** was tested at both −21°C and −196°C in EtOH (1 mCi/mL).

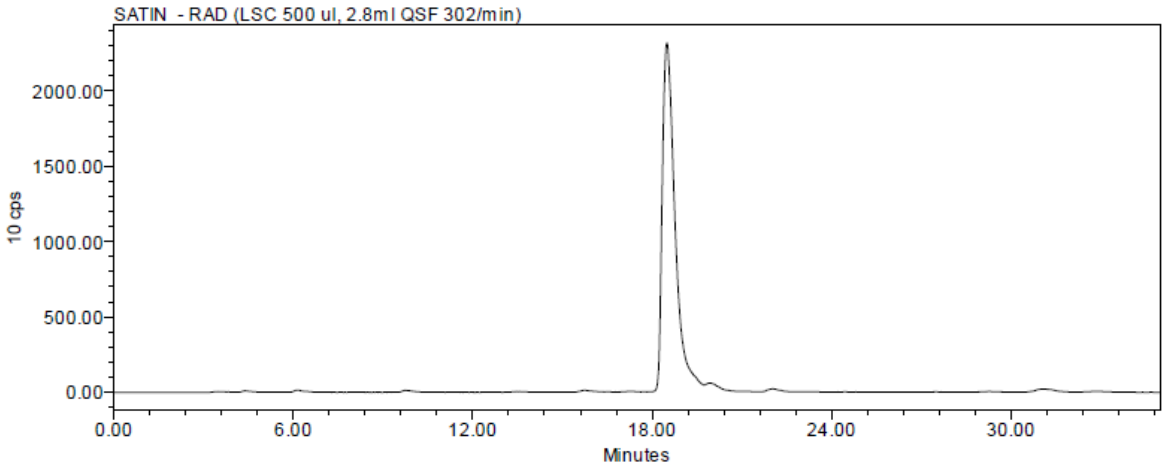
Stability HPLC radiochromatogram of [^{3}H]-**1** in EtOH at −196°C (2 months after formulation / 1 mCi/mL). Radiopurity >94%.



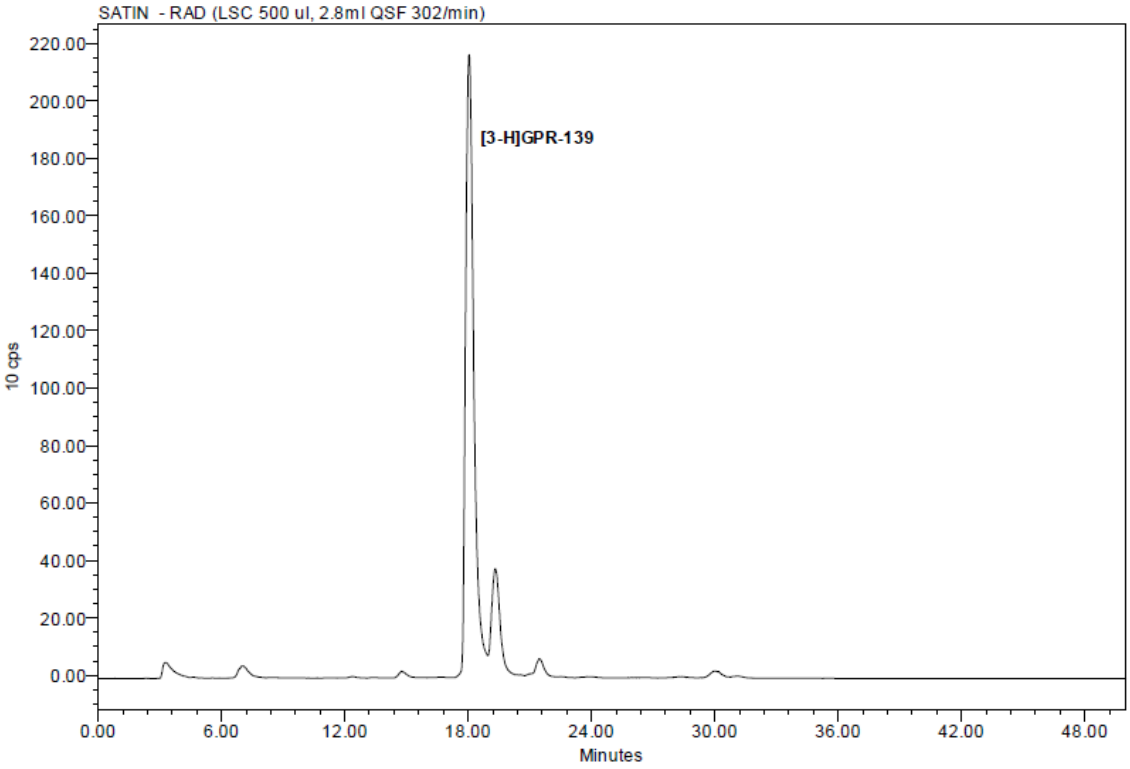
Stability HPLC radiochromatogram of [^{3}H]-**1** in EtOH at −196°C (10 months after formulation / 1 mCi/mL). Radiopurity >90%.



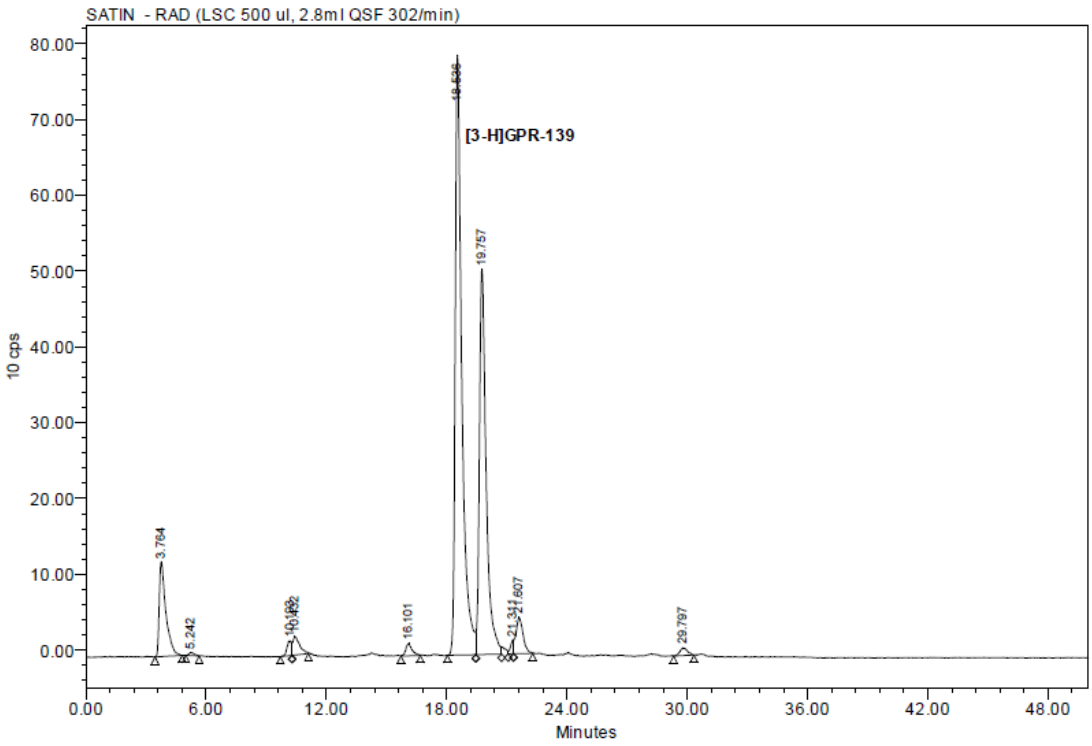
Stability HPLC radiochromatogram of [^{3}H]-**1** in EtOH at −21°C (4 weeks after formulation / 1 mCi/mL). Radiopurity >92%.



Stability HPLC radiochromatogram of [^{3}H]-**1** in EtOH at −21°C (2 months after formulation / 1 mCi/mL). Radiopurity >81%.



Stability HPLC radiochromatogram of [^{3}H]-**1** in EtOH at −21°C (10 months after formulation / 1 mCi/mL). Radiopurity >50%.



PHARMACOLOGICAL CHARACTERIZATION OF [^{3}H]-1

GPR139 knockout mice

The GPR139 knockout mouse, Gpr139$^{tm1(KOMP)Vlcg}$, was purchased from The Knockout Mouse Project, KOMP Repository, University of California[2] (https://www.komp.org/geneinfo.php?geneid=61381) and bred at Charles River Laboratories, Inc, Germany. Animals were genotyped at birth or at weaning by Charles River Laboratories using the following 3 primers: 1) GPR139 forward 5'-GTTCTGAGGCGGGAGCTGAG-3', 2) GPR139 reverse 5'-GCACACCCACAAGTCACAATG-3' and 3) GPR-5'-LacZ GCTGGCTTGGTCTGTCTGTCCT-3'. Animals were group-housed by gender with littermates under standard conditions. Ethical permission for the breeding of the GPR139 KO mice and litter mates was granted by the Danish Animal Experiments Inspectorate (2015-15-0202-00019).

Autoradiography

To determine GPR139 binding using [^{3}H]-**1**, brains were sliced in 12 µm coronal sections (starting from approx. interaneural 4.98 mm and bregma 1.18 mm), mounted on Super Frost Plus slides (ThermoScientific) and stored at −80 °C until further processing. The non-specific binding was determined using unlabelled **1**. Briefly, sections (4 sections per slide) were allowed to thaw for 1 hour at room temperature (RT) and then pre-incubated with either 50 mM Tris buffer, pH 7.4 or 50 mM Tris buffer, pH 7.4 + 10 µM **1** for 30 minutes at RT under constant gentle shaking. Sections were then incubated for 60 minutes at RT using the same buffers containing either 1 nM or 10 nM of [^{3}H]-**1**. Following incubation, slides were washed 2 × 20 seconds in ice-cold 50 mM Tris buffer, pH 7.4, 1 × 20 seconds in ice-cold milli-Q H_2O, and dried for 1 hour under a gentle stream of air. All sections were placed at 4 °C overnight in a fixator containing

paraformaldehyde vapour and then put in an dessicator box for 3 hours before slides and a [^{3}H]-microscale (batch 21A, February 2014, GE Healthcare) were exposed to a BAS-TR2040 Imaging Plate (Science Imaging Scandinavia AB) for 3 days. Finally, the imaging plate was scanned on a CR-35 Bio scanner (Dürr Medical). The [^{3}H]-microscale (nCi/mg TE) was used to quantify the results using the program ImageJ V.1.49j.

Cell culture

CHO-k1 cell line stably expressing human GPR139 (CHO-k1 GPR139) and a CHO-k1 background cell line were used in this study. The cells were grown in Dulbecco`s modified eagle medium (DMEM) F12-Kaighn's (Gibco, 21127) supplemented with 10% dialyzed fetal bovine serum (Gibco, 26400, United State origin), 1% GlutaMAX^{TM}-I (100X) (Gibco, 35050), and 100 units/mL penicillin and 100 µg/mL streptomycin (Gibco, 15140). For the CHO-k1 GPR139 1.0 mg/mL geneticin (Invitrogen, 1181103) was added to the growth medium.

RNA extraction and cDNA synthesis

GPR139 mRNA level was determined in the CHO-k1 GPR139 and CHO-k1 cell line. Similarly, RNA was extracted from striatum and hypothalamus from both GPR139 WT and KO mice. RNA extraction was performed using PureLink® RNA mini kit (Ambion) and treated with DNase using Turbo DNA-free kit (Ambion), according to the manufacturer's protocols. The concentration of isolated RNA was measured at a NanoDrop 2000 spectrophotometer (Thermo Scientific). The reverse transcription was performed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) on a standard PCR machine (25 °C for 10 minutes, 37 °C for 2 hours, 85 °C for 5 minutes) and stored at −20 °C until further processing.

Quantitative PCR

Quantitative PCR (qPCR) was performed in 96-well plates (Agilent) mixing PerfeCTa SYBR Green FastMix (Quanta), nuclease free water (Qiagen), and primers (TAG Copenhagen A/S, for primer sequences, see Table 1). The PCR was performed applying an initial denaturation step of 95 °C for 30 seconds, followed by 40 cycles of 5 seconds at 95 °C, 15 seconds at 60 °C and 10 seconds at 72 °C. To assure single-product amplification, a dissociation curve analysis was performed consisting of 60 seconds at 95 °C, 30 seconds at 55 °C and 30 seconds at 95 °C. The qPCR was performed using the Agilent Mx3005P qPCR system (Agilent Technologies) and the corresponding MxPro software was used to determine the Ct values. The ΔCt values were calculated using $2^{(\text{B2M Ct} - \text{GPR139 Ct})}$ according to Schmittgen & Livak.[3]

mGPR139 forward	5'-CCCACCTCGCTGCGAATAG-3'
mGPR139 reverse	5'-AGATATTTGCTGGTAACCCGA-3'
hGPR139 forward	5'-TGCCTCGGTTTACCAGCAAAT-3'
hGPR139 reverse	5'-AAGAGGACCAAGATGTCGGC-3'
mB2M forward	5'-CAGAAAACCCCTCAAATTCAAGTAT-3'
mB2M reverse	5'-AATTCAGTGTGAGCCAGGATATAGA-3'
cho B2M forward	5'-ACGGAGTTTACACCCACTGC-3'
cho B2M reverse	5'-CAGACCTCCATGATGCTTGA-3'

Table 1. Sequences of primers used for qPCR.

Membrane preparation

Membranes were prepared from the CHO-k1 GPR139 and CHO-k1 cell line. Cells were grown to 100% confluency, then washed twice in ice-cold PBS, scraped off in HME-buffer (25 mM HEPES, 2 nM $MgCl_2$, 1 mM EDTA, pH 7.4) and transferred to pre-chilled Eppendorf tubes and centrifuged for 10 minutes at 14,000 *x g* at 4 °C. The supernatant was discarded and the pellet was re-suspended in ice-cold HME-buffer. The membranes were snap-frozen in an ice-bath (96% ethanol + dry ice) and stored at −80 °C until used. The tubes were thawed quickly (< 1 minute), placed on ice and two pre-cooled zirconium beads (1 mm) were added per tube. Membranes were homogenized for 20 seconds (full speed) using a Bulletblender (NextAdvance). The beads were removed and the samples were centrifuged for 10 minutes at 14.000 *x g* at 4 °C. The supernatant was discarded, and the pellet was re-suspended in ice-cold incubation buffer A) 50 mM Tris buffer, pH 7.4; B) 50 mM Tris, 2.5 mM $CaCl_2$, pH 7.4 or C) 20 mM HEPES, 100 mM NaCl, 10 mM $MgCl_2$, pH 7.4). The protein concentration was measured using Bio-Rad Protein Assay (Bio-Rad).

Pre-incubation of filters for membrane binding

The GF/C unifilters were either not pre-incubated or pre-incubated in 1 hour in 0.2% bovine serum albumin or polyethylenimine. Pre-incubation or no pre-incubation of the GF/C unifilters gave no significant difference in the filterbinding (Figure S1). 50 nM [^{3}H]-**1** in buffer C did, however, give relatively high binding with no pre-incubation of the filter, but because the binding of 50 nM [^{3}H]-**1** in buffer C on CHO-k1 membranes was equally high this was not considered specific (data not shown).

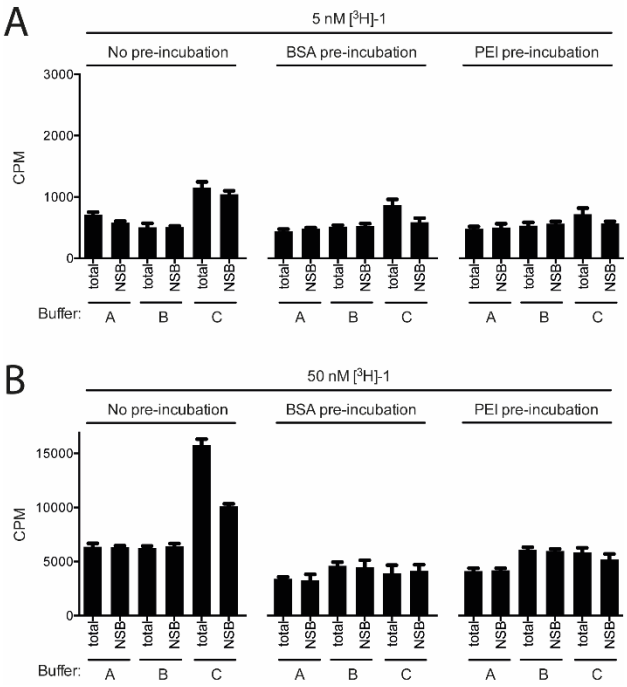


Figure S1. Membranes from CHO-k1 GPR139 cells were incubated with **A:** 5nM or **B:** 50nM [^{3}H]-**1** either in the absence (total) or presence (NSB) of 4μM cold **1** in either A) 50 mM Tris buffer, pH 7.4; B) 50 mM Tris, 2.5mM $CaCl_2$, pH 7.4; or C) 20 mM HEPES, 100 mM NaCl, 10 mM $MgCl_2$, pH 7.4.

Membrane binding assay

[^{3}H]-**1** binding was performed in 96-well plates using the filtration method. The CHO-k1 GPR139 and CHO-k1 membranes were dissolved in incubation buffer A, B or C (see above). 20-22 μg protein/well were incubated with 5 nM or 50 nM [^{3}H]-**1** either in the absence (total) or presence (NSB) of 4 μM **1** in triplicate at 0-4 °C for 2 hours. After rapid filtration through GF/C unifilters, radioactivity was measured using a Packard TopCount NXT Microplate Scintillation Counter (PerkinElmer).

Data analysis

Autoradiograms were analysed with ImageJ V.1.49j (http://imagej.nih.gov/ij). qPCR data was analysed using MxPro software. All data was plotted using GraphPad Prism 6.0 (GraphPad Software Inc.).

COMPUTATIONAL METHOD

The molecular descriptor calculations (i.e. clogP and logS) of **1** and JNJ-63533054 were performed using Molecular Operating Environment (MOE) software (version 2014.0901), developed by Chemical Computing Group, Inc. (Montreal Canada).[4] The compounds were created in ChemBioDraw Ultra 14.0.0.117, copied to MOE and imported in a database. Subsequently, the clogP and logS were calculated with the descriptor module in MOE.

REFERENCES

1. F. Shi, J. K. Shen, D. Chen, K. Fog, K. Thirstrup, M. Hentzer, J. J. Karlsson, V. Menon, K. A. Jones, K. E. Smith and G. Smith, *ACS Med. Chem. Lett.*, 2011, **2**, 303-306.
2. K. C. Lloyd, *Ann. N. Y. Acad. Sci.*, 2011, **1245**, 24-26.
3. T. D. Schmittgen and K. J. Livak, *Nat. Protoc.*, 2008, **3**, 1101-1108.
4. MOE, version 2014.0901; Chemical Computing Group (CCG): Montreal, Canada; http://www.chemcomp.com/MOE-Molecular_Operating_Environment.htm.